National Institute of Arthritis, Metabolism, and Digestive Diseases.
100
Report of program activities.

NIAMDD

ANNUAL PROJECT REPORTS

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NIAMDD ANNUAL REPORT

Fiscal Year 1980

OFFICE OF THE DIRECTOR

In 1980 the National Institute of Arthritis, Metabolism, and Digestive Diseases begins its fourth decade of serving the American people through the conduct and support of biomedical research.

The programs of NIAMDD deal with a wide range of categorical disease responsibilities representing health problems of great severity and magnitude. These diseases include arthritis and connective tissue diseases; skin diseases, such as psoriasis; diseases of the bone and musculoskeletal system; diabetes, cystic fibrosis, and other metabolic and endocrine disorders; digestive diseases; nutritional disorders; diseases of the kidney and urinary tract; and blood diseases. The urgency of the efforts necessary to reduce the toll of premature death, disability, and human suffering resulting from these afflictions is intense.

Institute responsibilities in many of these areas have expanded greatly as a result of special legislation concerning arthritis, diabetes, and digestive diseases. This legislation has resulted in ambitious plans of action, which have begun to be implemented in recent years. The Institute is responsible for conducting, supporting, and fostering research on diseases that affect all ages and sexes, all ethnic groups and strata of society, research of fundamental importance to virtually every citizen in our country.

Rheumatic Diseases

More than 31 million people in the U.S., for instance, are afflicted with one or more of the 100 types of arthritis and other rheumatic and connective tissue diseases, at a cost to the nation of over $14 billion annually. The national arthritis program conducted and supported by NIAMDD is continuing investigations of abnormal connective tissue activation in rheumatic diseases, the joint-eroding effects of collagenase and lysosomal enzyme release and of immune complexes in rheumatoid arthritis, immunologic derangements in systemic lupus erythematosus (SLE), the mechanics of normal and arthritic joint action and of prostheses (artificial joints), and many other related subjects.

Both the National Arthritis Act (P.L. 93-640) and the recommendations of the National Commission on Arthritis and Related Musculoskeletal Diseases called for intensified action to apply known treatments for arthritis, to teach necessary professional skills, to demonstrate and stimulate prompt and effective application of available knowledge for treatment of patients, and to develop new knowledge through Multipurpose Arthritis Centers. This NIAMDD program has grown to a current level of 24 Centers in 18 states, from New Hampshire to Hawaii. Another special

initiative, the NIAMDD Program for Cooperative Systemic Studies in the Rheumatic Diseases, continues to accumulate valuable clinical data as a basis for improved patient care. This program involves a consortium of 11 clinics, directed by a coordinating center at the University of Utah.

Musculoskeletal and Skin Diseases

The Institute also supports a broad program of research in orthopedics and bone diseases, including basic and clinical studies of skeletal tissues under normal and disease conditions. There are projects on calcium metabolism and bone formation, fracture healing and fixations, bone transplantation and preservation of skeletal tissue, prostheses (such as artificial joints) and relevant biomaterials, biomechanics and bioelectric phenomena, musculoskeletal dynamics and physiology of normal and abnormal gait. The areas of osteoporosis and osteogenesis imperfecta are receiving special program emphasis. The metabolism of cartilage is being studied at various centers throughout the country. A portion of funding in the fracture areas has been used to investigate improved bone healing through new methods of electrical stimulation.

Studies to determine research needs and priorities in the field of dermatology have been conducted, and the final report of this analysis, performed under contract at the University of Washington, was published during the past year. The Coordinating Committee of this major effort has also prepared summary statements, including the appraisal of current manpower resources, needs for additional research personnel, and an appraisal of technological development in this field. The plan of the Committee, setting forth priorities for research, was published in the Journal of Investigative Dermatology.

Diabetes and Metabolic Diseases

Enactment in 1974 of the National Diabetes Mellitus Research and Education Act has led to an unprecedented research attack on diabetes by NIAMDD, as the lead agency for other NIH components and Federal agencies in diabetes research. Since this new initiative began, the Institute has strengthened existing programs and undertaken many new ones. Eight Diabetes Research and Training Centers have now been established. Each conducts research into the causes, diagnosis and management of diabetes and its complications, and trains professionals for diabetes research and management of diabetic patients. The Centers also serve as focal points for effective transmission of research advances to health professionals and for the translation of these advances into improved care for patients. In addition, the NIAMDD supports three Diabetes-Endocrinology Research Centers, which conduct ongoing biomedical research. Research areas of special interest recently have been islet cell transplantation, insulin delivery systems, and epidemiology.

Diabetes is one of a broad spectrum of metabolic diseases and inborn errors of metabolism investigated through NIAMDD support. Cystic fibrosis (CF), a lethal inherited metabolic disorder, affects one infant in every

2,000 live births and costs the nation more than $150 million annually in treatment alone. The Endocrinology, Metabolic Diseases and Resources Programs Branch, established last year, serves as the focus for NIAMDD activities related to CF. The Institute directed a major effort during the past year toward increasing awareness among investigators of the needs and opportunities in CF research. This intiative resulted from a series of recommendations in the report entitled Cystic Fibrosis: State of the Art and Directions for Future Research Efforts. This valuable, comprehensive document, prepared through joint sponsorship with the National Heart, Lung and Blood Institute, involved the cooperation of a broad spectrum of scientists in relevant disciplines and was completed by the Cystic Fibrosis Foundation (acting under a contract from both Institutes) in fiscal year 1978. Because of these initiatives, the number of applications received for support of CF investigations has nearly tripled, resulting in an approximate 50 percent increase in the amount of support in the NIAMDD cystic fibrosis program.

Digestive Diseases and Nutrition

Digestive diseases hospitalize more Americans than any other group of disorders. They affect more than 20 million people, and their economic cost in direct medical care is estimated to be $17 billion a year and another $35 billion in lost work and wages. The NIAMDD digestive diseases program encompasses the full spectrum of research of organ systems associated with the gastrointestinal tract, including salivary glands, esophagus, stomach, pancreas, liver, gallbladder, and small and large intestines. Research activity is concerned with basic aspects of anatomy, histology, physiology, endocrinology, and biochemistry of the digestive tract in health and disease states. Future efforts will be directed toward implementing the long-range plan recommended by the National Commission on Digestive Diseases. The Commission dealt with a wide range of problems, including incidence and duration of digestive diseases, the mortality rates resulting from such illness, and the social and economic impact of these disorders. The NIAMDD is performing a central role in the implementation of many recommendations, and is working to fulfill them within the framework of existing fiscal and manpower resources.

The NIAMDD nutrition program continues to stress the following priorities: study of human nutritional requirements and factors that influence these requirements; use of new technologies in the study of the function of nutrients; studies on iron nutrition, metabolism, and deficiency; requirements and metabolic roles of trace minerals; basic studies on dietary fiber; research on the interaction of nutrition, immune competence, and infection; increased support for obesity research with the aim of prevention; and studies on supportive nutrition for hospitalized patients through Clinical Nutrition Research Units.

Since 1965, the malnutrition panel of the U.S.-Japan Cooperative Medical Science Program has served as the primary vehicle through which international nutrition research activities are identified for support.

The U.S. Malnutrition Panel has continued to support research relating to the nutritional health problems of Southeast Asian people; many of these are of universal scope and of importance to other populations including that of the United States. The objectives of this program as they are now defined are to study: (1) the influence of environmental and host factors on nutritional requirements; (2) health significance and methods of preventing iron deficiency; (3) interaction of nutrition, immune competence, and infection; (4) the effects of nutrition on physical and mental development, behavior, physical capability, and work performance; and (5) the health consequences of different dietary patterns and food intake.

Kidney and Urologic Diseases

The kidney and urologic diseases program represents a significant portion of the overall NIAMDD research effort. The many patients with chronic uremia and end-stage kidney disease represent but one facet of these diverse disorders. Investigators supported by NIAMDD are seeking new methods of preventive therapy, earlier diagnosis, and more effective treatment through expanded knowledge and understanding of the basic mechanisms and causes of a host of disorders. Areas of investigation include the study of glomerulonephritis, nephrosis, interstitial nephritis, kidney and bladder stones, polycystic kidney disease, diabetic nephropathy, analgesic-associated and other toxic nephropathies, immune disorders of the kidney, benign prostatic hyperplasia, and numerous other diseases.

Three sub-programs have been established in the Institute's extramural program related to the urinary tract: the Renal Physiology and Pathophysiology Program, the Urology Program (including urolithiasis), and the Chronic Renal Disease Program. In addition to these, the Hematology Program focuses research on the fundamental structure, function, and metabolism of blood components. An evaluation of this program in relation to research needs was undertaken last year in cooperation with other Institutes of the NIH with blood programs, and a final report of this significant study should be available by 1981.

During 1980, the kidney and urologic disease program of the Institute held a Conference on Chronic Renal Disease to enable scientists to obtain an overview of the state of the art of research in the pathophysiology of uremia, dialysis, and transplantation. These meetings included sessions on renal osteodystrophy, vitamin D analogs, parathyroid hormone, cardiovascular complications of chronic renal disease, neurologic complications of uremia, drugs and renal failure, and complications occurring during dialysis therapy and transplantation, with emphasis on immunosuppressive therapy. The meetings provided a valuable opportunity for profitable discussions among scientists engaged in assessing current approaches to research and in planning for the most effective use of limited resources in reducing the morbidity and mortality of chronic renal disease in the United States. A similar opportunity to review the state of the art will be provided at a Conference on Nephrotoxic Mechanisms of Drugs and

Environmental Toxins, soon to be sponsored jointly by the NIAMDD and the National Institute of Environmental Health Sciences. Scientists at these meetings will explore the current state of knowledge and concerns relating to such areas as chronic interstitial nephritis, environmental toxicities and hazards, immunopathological mechanisms, nephrotoxicity of antibiotics, and renal failure models.

Training for Research

Across all of its categorical programs, the Institute continues to be concerned about adequate support of training to provide research investigators needed for the future. In particular, there is concern about continuing development of qualified clinical investigators who bring to the study of disease in human beings the competence and sophistication of so-called "basic scientists" (Ph.D.s) in bio-medicine and who make the necessary bridge between laboratory science and sensitive, accurate observation of disease processes in patients. Recently it has become clear that young M.D.s are less interested in scientific study than in years past; consequently, NIAMDD has joined in the NIH Short-Term Training Program, designed to reawaken the interest of medical students in research. Other mechanisms will need to be devised to ensure the necessary quality and quantity of clinical investigation.

Programs for Minorities

The Institute has continued its commitments to provide training for its personnel. Short-term training opportunities, for example, have been provided to more than 115 secretarial-clerical employees, to supervisory-managerial personnel, and to 106 members of the professional staff. The EEO Advisory Committee is currently planning appropriate Supervisory/EEO training for its members.

The NIAMDD EEO Advisory Committee meets about seven times a year to discuss issues and concerns and to formulate recommendations to meet EEO objectives as spelled out in the Institute's Affirmative Action Plan. This year, the Committee participated in a two-day training session designed to provide its members with an historical perspective of EEO and an understanding of the human resource management concept, which relates EEO to personnel management.

The NIAMDD Advisory Council this year approved funds for support of the intra-agency agreement of the Minority Biomedical Support Program with the Division of Research Resources and the agreement for the support of the Minority Access to Research Careers Program with the National Institute of General Medical Sciences. The intent of these programs is to increase the number of minority scientists engaged in biological research/health sciences. In addition, the Council approved funds for the support of 20 high school students at centers around the country under the Research Apprenticeships for Minority High School Students Program announced by President Carter on October 31, 1979, and directed by the Office of Science and Technology Policy. In addition, this year

the Institute initiated a new Career Program to provide further upward mobility opportunities for lower-graded employees.

The institutional initiatives described in this report, combined with basic and applied research efforts, are expected to yield great benefits in disease prevention, lives saved, and in extension of human life of maximal quality and productivity.

Annual Report of the Laboratory of Nutrition and Endocrinology
National Institute of Arthritis, Metabolism and Digestive Diseases

Membrane Regulation Section

Mechanism of Action of Hormones and Guanine Nucleotides on Adenylate Cyclase Systems

We have continued the use of high energy irradiation analysis for determining the size and composition of adenylate cyclase systems under various "ground-state" and ligand (hormones, guanine nucleotides, and fluoride)-induced states of the enzyme. Last year we reported that the ground state of the liver enzyme consists of an organized oligomeric complex of glucagon receptors (R) and GTP-regulatory units (N). We have proposed that this oligomeric complex is constructed such that the receptor inhibits the interaction of N with GTP; hormone binding to the oligomer promotes binding of GTP to N with resultant breakdown of the oligomers to smaller units that functionally link with the catalytic unit (C) of adenylate cyclase. From studies of the adipocyte adenylate cyclase system, which is activated by several different hormones, we deduced that the same mechanism applies to activation of this system. Based on the finding that the target size of the GTP-inhibitory process in adipocytes is larger in its ground state than the ground state target of the hormone-sensitive GTP-stimulatory process, we surmised that these processes are composed of different structures and that both types of structures may be composed of an organized array of receptor and GTP-regulatory units.

During this past year we demonstrated that the catecholamine-sensitive adenylate cyclase system in turkey erythrocytes has properties very different from liver and adipocyte adenylate cyclases. The turkey system is activated by hormone plus Gpp(NH)p but not by the naturally occuring nucleotide activator, GTP. This system also binds GDP tightly at the N unit. Addition of hormone (isoproterenol) and GMP (a non-activating nucleotide) results in release of bound GDP. Clearance of the N unit of GDP results in two significant changes in the enzyme system: (i) Binding affinity of the beta-receptor for agonists is enhanced; and (ii) Occupation of the cleared N unit by Gpp(NH)p results in activation of the enzyme. These findings provided the first definitive evidence that the N unit responsible for activation of adenylate cyclase is the same unit that is linked to the beta adrenergic receptor. These findings also suggested that the various components (R,N, and C) are pre-assembled in the erythrocyte membrane as the holoenzyme (RNC) complex. The results of target analysis of the erythrocyte system provided further evidence for this contention. Evaluated were the sizes of the enzyme system with MnATP as substrate (a measure of the size of the catalytic unit), when activated by fluoride ion (a measure of NC), and when activated by isoproterenol and Gpp(NH)p (a measure of RNC). The size of C was 100K daltons, NC was 230K, and RNC was 315K for the ground state and pre-activated forms of the enzyme. Thus, the large oligomeric structure found in liver and adipocyte membranes is absent in turkey erythrocyte membranes; the size of the ground state enzyme of the turkey is the same of that of the liver and adipocyte systems when activated by various effectors (hormones, guanine nucleotides,

and fluoride ion). We concluded that the turkey system represents an "end-stage" version of the liver and adipocyte systems in that it may have been formed from the oligomeric RN complex by prior activation prior to or during isolation of the membranes. The tightly-bound GDP may be the result of a GDP-induced inhibited form of the holoenzyme. Addition of both hormone and an exogenous nucleotide is required to release the inhibitory constraints imposed by bound GDP. Based on the increment (130K) in target size of the fluoride-activated enzyme over that of the MnATP-dependent form of the enzyme (C), it would appear that the N unit cannot exist solely of the 44K dalton unit which is known to react with cholera toxin and NAD and which represents the site of guanine nucleotide action. The increment (90K) in target size for the hormone/Gpp(NH)p-activated enzyme (RNC) over that of the NC unit proved to be identical to the target size (96K) of the β-adrenergic receptor measured by the binding of the antagonist, ^{125}I-hydroxybenzylpindolol. Thus, measured either by the actions of the agonist (isoproterenol) or of an antagonist, the β-adrenergic receptor has the same functional size. Since 90-96K for the functional size of the receptor is considerably larger than the reported subunit sizes (34-39K) of the β-receptor, we conclude that the receptor as well as the N unit in the turkey membrane consists of multiple heterologous subunits having differing regulatory functions. This possibility gives a structural basis for the complex kinetic features of the turkey system and opens the way for a better understanding of how hormones operate on these systems. (P.M. Lad, T. Nielsen, W. Schlegel, M.S. Preston, M. Rodbell).

Studies of Hormone Responses in Cultured Cells

An established kidney cell line (MDCK) contains an adenylate cyclase system that is responsive to vasopressin, glucagon, catecholamines, and prostaglandins (PGE_1), as measured by increases in cyclic AMP production. We have found the following: (i) A cloned MDCK cell transformed by Harvey murine sarcoma virus shows selective loss of glucagon responsiveness and a reduction in vasopressin sensitivity. By contrast, responses to catecholamine and prostaglandin remain unchanged; (ii) Addition of sodium butyrate to the culture medium restores glucagon sensitivity; (iii) Disappearance and reappearance of glucagon receptors are partly responsible for the changes in glucagon sensitivity; (iv) Butyrate-induced glucagon sensitivity differs from the untransformed parent cell in that the efficacy of response is reduced; this aberration seems to be intrinsic to viral transformation and specific for the glucagon response; (v) Tunicamycin, a glycosylation inhibitor, blocks the induction of glucagon response by butyrate, suggesting that the glucagon receptor is a glycoprotein (M. Lin).

The GTP Inhibitory Process

Our earlier studies established that the fat cell adenylate cyclase was subject to dual GTP-mediated regulation; a stimulatory process which permitted lipolytic hormones to stimulate activity and an inhibitory process, which promoted inhibition by adenosine. We have added to our previous evidence for the non-identity of the GTP-regulatory proteins mediating these two processes by the technique of target size analysis. These studies indicate that the proteins mediating the inhibitory process are larger aggregates than those mediating stimulation.

We have discovered that similar dualistic regulation occurs in brain cortex and caudate nucleus. In the former case adenosine promotes the inhibition, whereas in the latter inhibition is promoted by opiates. Common characteristics are shared by each of these systems in that there is a total dependence on the second (GTP-mediated) process, for the observation of these effects, sodium ions generally amplify the effects and Gpp(NH)p will not promote the inhibition. Awareness of these regulatory circuits should permit the detection of interactions of neurotransmitters/hormones in many systems.

Currently we are attempting to apply further biochemical techniques to the elucidation of the structural composition of such complex regulatory assemblies. (D.M.F. Cooper, C. Londos and W. Schlegel).

Actions of Adenosine and Antilipolytics

We established previously that plasma membranes from most cells contain adenosine receptors that are linked to adenylate cyclase, and that the receptor that mediates activation in Leydig tumor cells and hepatic membranes is pharmacologically distinct from the receptor that mediates inhibition of the fat cell cyclase. Upon examination of additional tissues, we now find that the pharmacological profiles established for the above cell apply generally. That is, the turkey erythrocyte and Y-1 adrenal tumor cell contain stimulatory adenosine receptors identical to those found in hepatic membranes, whereas brain cortex contains an inhibitory receptor identical to that found in fat cells. Thus, adenosine receptor subclasses are distinguished both by the specific reaction elicited with adenylate cyclase and by the potency series exhibited with selected receptor agonists.

The finding that adenosine, a potent antilipolytic agent inhibits the fat cell adenylate cyclase directly led us to examine the mechanisms by which adenosine and other antilipolytic agents inhibit cyclase, and to compare the effects of these agents on adenylate cyclase with their effects on the lipolytic response. Antilipolytics (adenosine, prostaglandins, and nicotinic acid) inhibit the fat cell cyclase by a GTP-dependent and Na^+-enhanced process. With the use of a wide variety of analogs of the antilipolytic agents, we have established that the receptors through which these agents mediate adenylate cyclase inhibition in purified adipocyte membranes are identical to the receptors through which they mediate the antilipolytic response. Moreover, adenosine, prostaglandins and nicotinic acid exert their actions through separate receptors, but share a common pathway since their effects are not additive. Fat cell adenylate cyclase catalytic activity and lipolysis in adipocytes represent a balance between stimulatory (lipolytic) and inhibitory (anti-lipolytic) forces. As with the lipolytic response in intact cells, we find that supression of cyclase by antilipolytics is limited to low activity states of the enzyme, and that excessive stimulation by lipolytic agents diminishes the effects of the antilipolytics. Thus, by both kinetic and pharmacological criteria, our findings on adenylate cyclase parallel the findings in intact cells. (C. Londos, D.M.F. Cooper and J. Wolff).

Developmental Biochemistry Section Summary

Physical studies of nucleosome core particle structure

In the past year, we have further refined knowledge of several aspects of the structure of the chromatin core particle. The state of histones H2A, H2B, H3 and H4 in high salt concentrations has been thought to mimic their state when associated with DNA in the nucleosome. While the latter is clearly an octamer, the presence of heterotypic tetramers in 2M NaCl has been reported by several groups; functional significance has been attached to the occurrence of such a tetramer in terms of both replication and transcription. Using high precision membrane osmometry, a method which circumvents many of the experimental difficulties in methods employed by others, we find that the histones in 2 M NaCl exist as an octamer in reversible equilibrium with a hexamer ($H3_2$-$H4_2$H2AH2B). No evidence for the presence of a heterotypic tetramer was found, leading us to suggest that the possible role of this entity in vivo be reexamined.

Calorimetric measurements allow dissection of the energetics of stabilization of a macromolecular structure. In calorimetric studies of core particle denaturation (in collaboration with J. Sturdevant, Yale University), we find that the free energy of stabilization of nucleosome DNA is about 66 kcal/mol greater than that for DNA alone. Taking into account the likely heat of protein denaturation and the temperature dependence of the transition enthalpy of DNA melting, it appears that this additional free energy is likly entropic in origin. This is consistent with theoretical considerations for a species where the primary stabilizing forces arise from electrostatic interactions.

In the past, study by ^{31}P NMR of core particles has provided evidence that kinked structures do not occur in the nucleosome DNA. We (in collaboration with H. Shindo, LCP) now confirm this with a core particle containing poly(dG-dC) which has a smaller line width than either native DNA- or poly(dA-dT)-containing particles. Additionally, we can demonstrate that the G-C containing particles do not have Z-form (for zig-zag) DNA, the right-handed, alternating double helical structure recently described by Wang et al., making it less likely that the suggestion that this DNA might exist in complexes of DNA with highly basic proteins in vivo is correct. Using this homogeneous DNA, we show that the Pohl-Jovin transition, likely from left-handed B-form to right-handed Z-form, is, somewhat surprisingly, an intramolecular reaction which does not require complete strand separation.

Role of histone H1 in chromatin structure

We have previously described the structure of the chromatosome, a nucleosome particle containing 160 base pairs of DNA, the octamer of inner histones, and one molecule of H1 (or H5). The particle contains two full turns of DNA around the histone octamer, stabilized by binding of H1 to the ends of the DNA; the protein is located outside the nucleic acid. Occurrence of this particle in vivo makes several predictions about the effect of H1 on chromatin reconstituted in vitro with or without H1. We have examined two of these this year.

The prediction can be made that addition of H1 to a reassociation system should lead to nucleosome spacing at longer intervals on the DNA than in its absence. Chromatin formed by salt gradient dialysis from the inner histones and DNA has a particle spacing corresponding to 145-150 base pairs of DNA; the material consists of close-packed core particles. Incubation of this material with H1 and poly-glutamic acid at 0.1 M NaCl leads to lengthening of the spacing to about 180 base pairs. This is the best lead available at this time to the question of how to faithfully reconstitute the second level of DNA organization in association experiments.

If the core particle contains 1.75 turns of DNA and the chromatosome contains 2.0 turns, the number of superhelical twists induced on closed circular DNA by formation of the two particles should differ. In experiments where SV40 DNA I° was associated with inner histones and then with H1 plus polyglutamic acid, it was found that the absolute magnitude of the linking number change per nucleosome increased significantly. A number of control experiments demonstrated that this change is not due to formation of new nucleosomes or due to direct interactions of H1 with non-nucleosomal DNA. Apparently H1 stabilizes a more complete wrapping of DNA around the histone octamer, consistent with the prediction from the chromatosome structure.

Transcription of a yeast gene

We are attempting to develop a system which will allow in vitro transcription of a specific gene sequence as chromatin. The system currently employed has the yeast trp1 gene inserted in pBR313 to form the plasmid pLC544. The gene sequence is known and the plasmid replicates in either E. coli or yeast. While not definitive, current results indicate that it is possible to obtain accurate initiation on the trp1 sequence using exogenous yeast RNA polymerase II and partially purified cytoplasmic factors. In order to develop methods for isolation of the plasmid as a minichromosome from its yeast host, we are isolating and characterizing the 2 micron plasmid which is a naturally occurring entity in many yeast strains.

Structure of the sea urchin sperm chromatin core particle

In beginning studies of chromatin changes at fertilization and during early to embryogenesis, we have characterized the core particle of sea urchin sperm. H2A and H2B in this particle differ strikingly in composition and sequence from the equivalent molecules in the adult. The particle bears many similarities to "typical" core particles from adult tissues; it contains 145 base pairs of DNA and a histone octamer, has similar sedimentation and circular dichroism properties, and undergoes expansion under the stress of low ionic strength. In contrast to other particles, the sperm core particle has a different melting profile; the reversible transition due to unwinding of 20 base pairs of DNA from each end occurs at a much higher temperatures near that required for total, irreversible denaturation of the nucleosome. DNAase I mapping indicates that several additional sites (vs. other core particles) are protected from the nuclease in the sperm particle. Thus, the presence of the variant forms of H2A and H2B leads to a modulation of the structure of the core particle. Since other variants of these two histones occur during the transition from zygote to gastrula in sea urchin, we anticipate that different types of core particle structure might exist at different phases of early embryogenesis.

Vitamin Metabolism Section

The major accomplishment of the past year has been the determination of the primary amino acid sequence of dihydrofolate reductase from chicken liver. Dihydrofolate reductase which is concerned with the conversion of the essential vitamin folic acid to the biologically active cofactor tetrahydrofolate has a central role in cellular proliferation. Thus the structure of this enzyme will be of considerable pharmacological and biochemical interest since dihydrofolate reductase is the receptor for the powerful and useful drug, methotrexate. This current accomplishment is based on previous studies from this laboratory which led to the development of affinity chromatographic procedures for the purification of relatively large amounts of homogeneous, cosubstrate-free, crystalline dihydrofolate reductase from chicken liver. Thus sufficient enzyme protein from liver, the main site of dihydrofolate reductase activity in animals, was now available for sequencing. The S-carboxymethylated protein was subjected to cleavage by cyanogen bromide which produced five fragments. Sequences and ordering of the cyanogen bromide fragments were established by means of automated sequencer analysis of the fragments and from smaller peptides generated by proteolysis with trypsin and staphyloccal protease. The complete amino acid sequence comprising the covalent structure of the single polypeptide chain consists of 189 residues of molecular weight 21651.

Since the nature of the binding of drugs to dihydrofolate reductase is of considerable importance to a rational approach to chemotherapy, the analysis of a model system for such interactions yields significant information. For example, Cibacron blue F3GA binds to dihydrofolate reductase from chicken liver and Lactobacillus casei in an 'electrostatic mode' and 'hydrophobic mode' respectively. Although the enzyme has a 'pseudo-dinucleotide fold', the blue dye does not bind at the proper nucleotide binding site to the enzyme from either species.

Salt (NaCl) acts as an activator for the reaction catalyzed by dihydrofolate reductase (from chicken liver) if NADPH is used as the coenzyme and as an inhibitor if NADH is used as the coenzyme. ^{35}Cl NMR studies reveal that this dichotomy in the kinetics is a result of the mutual exclusivity of chloride and NADPH binding at the active site of the enzyme while chloride and NADH binding to the enzyme are mutually inclusive. Additional studies on enzyme substrate interactions have involved glutamate dehydrogenase. The reaction catalyzed by bovine L-glutamate dehydrogenase is subject to allosteric activation by ADP. We have measured the thermodynamic parameters of the formation of the various possible binary and ternary complexes formed between the enzyme, NADPH, and either ADP or its analogs. The data are interpreted in terms of "interaction parameter", the differences between the thermodynamic parameters of the formation of a ternary complex and the sum of those of the two related binary complexes. Both adenosine and ATP appear to loosen the binding of NADPH by simply preventing a subsite interaction of NADPH. AMP appears to have only minor and probably secondary effects. The negative effect of the binding of ADP on that of NADPH, however, involves the formation of a new interaction, which is exothermic, entropy compensated, has a moderately large negative ΔC_p, and does not occur in either binary complex.

Regulation of Lipoprotein Lipase and Hormone-sensitive Lipase in 3T3-L1 Fat Cells

During the past two years we have established that the mouse 3T3-L1 fibroblast cell line provides an excellent model system for studying hormonal regulation of lipoprotein lipase activity in adipocytes. Insulin was found to be the most important hormonal factor in expression of the fatty phenotype, development of lipoprotein lipase activity and deposition of triacylglycerol. In mature cells insulin has 3 distinct actions in the regulation of lipoprotein lipase: 1) It stimulates gene expression and RNA synthesis independently of effects on hexose and amino acid transport. 2) It stimulates hexose transport which is essential for expression of nuclear effects of insulin at the translational level. 3) It stimulates release of lipoprotein lipase from cells by a process that is independent of energy and uninhibited by colchicine and cytochalasin.

We recently found that antibodies to human insulin-receptors (Anti-R) mimicked the effects of insulin on lipoprotein lipase activity in mature 3T3-L1 fat cells. Anti-R also blocked the effects of insulin on the cells, suggesting that Anti-R and insulin both act through the same mechanism, and that metabolites of insulin are not "second messengers" internalized to act at nuclear sites, as proposed by others. (These findings were made in a collaborative study with Drs. E. Van Obberghen, C.R. Kahn, F.A. Karlsson, and C. Grunfeld of the Diabetes Branch of NIAMDD).

We have also studied the regulation of hormone-sensitive lipase in 3T3-L1 fat cells. This lipase hydrolyzes intracellular triacylglycerol to fatty acids and glycerol for release by fat cells, whereas lipoprotein lipase acts on exogenous triacylglycerol to provide fatty acids for synthesis of triacylglycerol in the cells. Hormone-sensitive lipase is activated by catecholamines and other hormones acting through specific receptors and adenyl cyclase. Hormone-sensitive lipase was measured by the amount of glycerol released by cells to the medium. Epinephrine and norepinephrine both stimulated hormone-sensitive lipase, but had very little effect on cellular lipoprotein lipase activity. Raising the molar ratio of fatty acids to albumin in the medium decreased hormone-sensitive lipase activity with minimal effect on lipoprotein lipase activity. Insulin and glucose enhanced both basal lipolysis and epinephrine-stimulated hormone-sensitive lipase. The results indicate that lipoprotein lipase and hormone-sensitive lipase are regulated independently in 3T3-L1 fat cells. (S.S. Chernick, P.M. Spooner and R.O. Scow).

Antibodies to Lipoprotein Lipase of 3T3-L1 Fat Cells

Lipoprotein lipase released to incubation medium by 3T3-L1 cells in response to insulin has been purified by affinity-chromatography with heparin-sepharose. Antibodies to the purified enzyme have been raised in rabbits. The antibodies neutralize the activity of lipoprotein lipase and will be used to study the induction of enzyme synthesis and the roles of insulin and glucose in this process. (S.S. Chernick, P.M. Spooner and R.O. Scow).

Transport of Lipolytic Products from Blood to Adipose Tissue by Lateral Movement in a Continuum of Cell Membranes

Electron microscopic studies of adipose tissue from suckling rats were undertaken to explore the concept that lipids are transported from blood to tissue by lateral movement in a membrane continuum. Our aim was to obtain structural data showing membrane continuity between capillary endothelium and parenchymal cells, and to determine if lipids derived from lipolysis are associated with this continuum. We used developing white adipose tissue of suckling rats because adipocytes in this tissue are rapidly taking up fatty acids from chylomicron triacylglycerol derived from dietary (milk) lipid. To explore the structure of developing adipose tissue we used a variety of fixation and preparation techniques, including osmium tetroxide alone, osmium tetroxide and glutaraldehyde together, and glutaraldehyde followed by post-fixation in osmium tetroxide, and we found that they provided complimentary information. We also developed a technique to visualize cell to cell contacts and cell junctions using tannic acid (MW 1700). Tannic acid applied to adipose tissue after aldehyde fixation does not cross cell membranes and it reacts with osmium tetroxide for form an electron-opaque complex which is limited to the extracellular space. With this technique we were able to discover channels within adipocytes and capillary endothelium which are open to the extracellular space and also areas of cell to cell contact. The channels in endothelial cells extended between the luminal and basal surfaces, and those in adipocytes extended from the cell surface to triacylglycerol droplets within the cells. Since these channels are open to the extracellular space, the membranes bounding them are continuous with the cell plasma membrane. Areas of contact between adipocytes excluded the tannic acid marker giving the appearance in sections of gap junctions. Areas of contact between capillary endothelium and adipocytes were also seen. In these areas basal projections of endothelial cells crossed through the underlying basement membrane material to adipocytes. Examination of cell membranes at these apposition areas (adipoendothelial junctions) showed that the external leaflets of adjacent plasma membranes came together to form a continuous leaflet. Our findings show that membrane continuity extends from the luminal surface of capillaries to the triacylglycerol droplet within adipocytes and that the external leaflets of cell membranes provide the interfacial continuum.

We further explored the possibility that lipids in transit between capillaries and adipocytes in developing white adipose tissue could be visualized with electron microscopic preparatory techniques. Previous studies on white adipose tissue of adult rats perfused with chylomicrons showed that hydrolysis of chylomicron triacylglycerol occurred after gluaraldehyde fixation when the tissue was incubated in buffer at 25°C. Electron microscopic studies on chylomicrons lipolyzed with lipoprotein lipase in vitro showed that amphipathic lipids could be seen as lamellar whorls with a banding pattern of 40 Å.

For our studies on lipolysis in developing white adipose tissue we used 8-12 day old rats, some of which were injected with chylomicrons and others were fasted overnight. Fat cells take up fatty acid from triacylglycerol in blood and release fatty acid from cell triacylglycerol during fasting. The former involves the action of lipoprotein lipase and the latter the action of

tissue lipase within fat cells. Adipose tissue from the animals was fixed in glutaraldehyde and then incubated in buffer solution for varying periods of time. Tissue for biochemical analyses was taken after fixation and after incubation. Tissue for morphological analysis was osmicated either immediately after aldehyde fixation or after incubation.

Biochemical analyses showed that hydrolysis of both chylomicron and cell triacylglycerol occurred in glutaraldehyde-fixed tissue when incubated, and morphological findings in these tissues showed that spiralling bilayered lamellae developed along the continuum between capillaries and fat cells. Morphometric studies of electron micrographs showed that the amount of lamellae increased with incubation time and that location of lamellae varied with both incubation time and metabolic state of the tissue donor. In tissue of chylomicron-injected fed rats lamellae developed first in capillaries and last in fat cells, whereas in tissue of fasted rats lamellae developed first in fat cells, near triacylglycerol droplets, and last at the luminal surface of capillaries. Lamellae were not found in tissue of chylomicron-injected fed rats if the tissue was incubated with insulin and glucose prior to fixation, indicating that the lamellae were composed of lipolytic products (fatty acids). Thus, in glutaraldehyde-fixed tissue from chylomicron-injected fed rats the lamellae represent accumulations of lipolytic products moving along the continuum during incubation from capillary lumina to fat cells, whereas in tissue from fasted rats they represent lipolytic products moving from fat cells to capillary lumina. We conclude that amphipathic lipids are transported in adipose tissue by lateral movement in a continuum of cell membranes. (E.J. Blanchette-Mackie and R.O. Scow).

Morphological Studies of Lipolysis and Lipid Transport in Rat Heart

The ultrastructure of hearts of 12-14 day old rats injected with chylomicrons was studied to test in this tissue our model for lipid transport from blood to tissue by lateral movement in a continuum of cell membranes. Initial surveys showed progressive accumulation of lamellar structures with incubation at 25°C in tissues previously fixed with glutaraldehyde. Biochemical analyses showed that chylomicron-triacylglycerol was hydrolyzed and that the products formed, fatty acids, accumulated in glutaraldehyde-fixed hearts when incubated at 25°C. These findings indicated that lipoprotein lipase activity was preserved in fixed tissues and that the lamellar whorls probably represented accumulations of amphipathic lipolytic products. Electron micrographs of tissue were analyzed stereographically to obtain data concerning location and amount of lamellar structures present in tissues under various conditions. The largest number of lamellae developed in tissues of fed rats injected with chylomicrons, while the smallest number developed in tissues of uninjected fasted rats. The lamellae were found along the proposed route of transport, in capillary lumina, within channels in capillary endothelial cells, in intercellular spaces, and within channels in ventricular muscle cells. Muscle cells of hearts from uninjected fed rats contained numerous lipid droplets, usually associated with mitochondria. Although the amount of lipid in cells was reduced in hearts from rats fasted overnight, lipid droplets were still found in these cells. Thus, accumulation of lamellae in hearts of uninjected rats, either fed or fasted, could result from hydrolysis of cell triacylgly-

cerol. In such cases, lamellae are found near mitochondria, a primary site of fatty acid utilization in heart. Lamellar whorls did not develop in heart tissue immediately fixed in osmium tetroxide after being removed from the animal. These findings demonstrate that chylomicron-triacylglycerol and also cell triacylglycerol can be hydrolyzed in glutaraldehyde-fixed heart, and the products formed move laterally in an interfacial continuum of cell membranes to sites where they form lamellar whorls as extensions of the continuum. We conclude that lipolytic products are transported in heart tissue by lateral movement in a continuum of cell membranes. (M.G. Wetzel and R.W. Scow).

In vitro Lipolysis of Human Chylomicrons by Lipoprotein Lipase

A study of the effect of lipoprotein lipase on human chylomicrons in vitro was carried out in collaboration with Drs. E. Schaefer and B. Brewer of NHLBI and Drs. G. Bengtsson and T. Olivecrona of Umeå, Sweden. Chylomicrons obtained from cannulated thoracic ducts of normolipidemic patients fasted overnight were incubated with purified bovine milk lipoprotein lipase in albumin/Tris solution, pH 7.4, at 37°C for 60 min. Biochemical, immunological and morphological analyses were performed on lipoprotein fractions of various densities resulting from lipolysis of chlomicrons. Apoproteins of chylomicrons prelabeled with radioiodine distributed primarily to the d 1.019-1.063 (g/ml), d 1.063-1.21 and d >1.21 fractions after >90% hydrolysis of chylomicron triacylglycerol. Acyl glycerol, cholesterol and apoprotein B were found predominantly in d 1.019-1.063 (LDL) fraction and apoproteins A-I and A-II in the d 1.063-1.21 (HDL) and d >1.21 fractions, while apoprotein C-III was fairly evenly distributed between the three more dense fractions, d >1.019.

Lipoprotein particles in the various fractions were negatively stained with phosphotungstate and examined with electron microscope. The findings, which confirmed our earlier observations on rat chylomicrons, showed progressive changes in configuration of chylomicrons undergoing lipolysis and appearance of remnants of surface film associated with variable amounts of core material. The resultant fragments were found in the denser lipoprotein fractions (d > 1.019), but they did not resemble the HDL particles found in corresponding fractions of normal human plasma; they were generally larger and more irregular in outline. We conclude they are fragments of surface components (proteins, phospholipid and cholesterol) from lipolyzed chylomicrons. (M.G. Wetzel and R.W. Scow).

Pharyngeal Lipase and Digestion of Dietary Fat

We have started a program to isolate pharyngeal lipase from serous glands of rat tongues. It was previously determined that pH 5.4 is optimal for lipolytic activity of the enzyme, measuring the amount of fatty acids released from triacylglycerol. The substrate used in the current experiments was an emulsion of trioleoylglycerol and tri[9,10-^{3}H]oleoylglycerol, stabilized with phosphatidylcholine, in glycerol. The assay procedure has been improved by incubating in medium containing 3.34 mM $CaCl_2$ and 17 mM taurodeoxycholate, instead of albumin. We have modified Belfrage-Vaughan's technique for extracting fatty acids from incubating medium by using 0.2 M glycine-NaOH, pH 12.4, for the aqueous phase. This assures a pH of 11.0-11.5 in the upper phase and

a consistent, maximal partitioning of fatty acids, 75-80%, into the upper phase.

The rate of lipolytic activity in extracts of lingual serous glands was linear for 2 min when assayed in medium containing 5.6 μmol of trioleoyglycerol and the amount of fatty acids produced was between 50 and 500 nmol. There was delay in onset of maximal activity when less than 50 nmol were formed, and partial suppression of activity when more than 500 nmol were formed. The mechanisms of these effects will be studied.

Homogenates of rat lingual serous glands were centrifuged at 850 x g and that supernatant used for isolating pharyngeal lipase. Preliminary studies had shown that separation of microsomes from this fraction did not increase enzyme activity/mg protein in either the microsomal fraction or the 106,000 x g supernatant. A ten-fold purification was obtained by precipitating with $(NH_4)_2SO_4$ and then desalting with Sephadex G-25. Filtration through Sephadex G-200 resulted in 96% recovery of protein and only 20% recovery of lipolytic activity in the void volume when eluted with 0.0125 M Tris-maleate buffer, pH 5.4. There was no increase in activity when the other fractions were added to the protein fraction. Much greater loss of protein and activity occurred when pH of the eluent was 7.4. We are now testing hydrophobic chromatography for use in isolating and purifying pharyngeal lipase. (R.B. Field and R.O. Scow).

Annual Report of the Laboratory of Nutrition and Endocrinology
National Institute of Arthritis, Metabolism, and Digestive Diseases

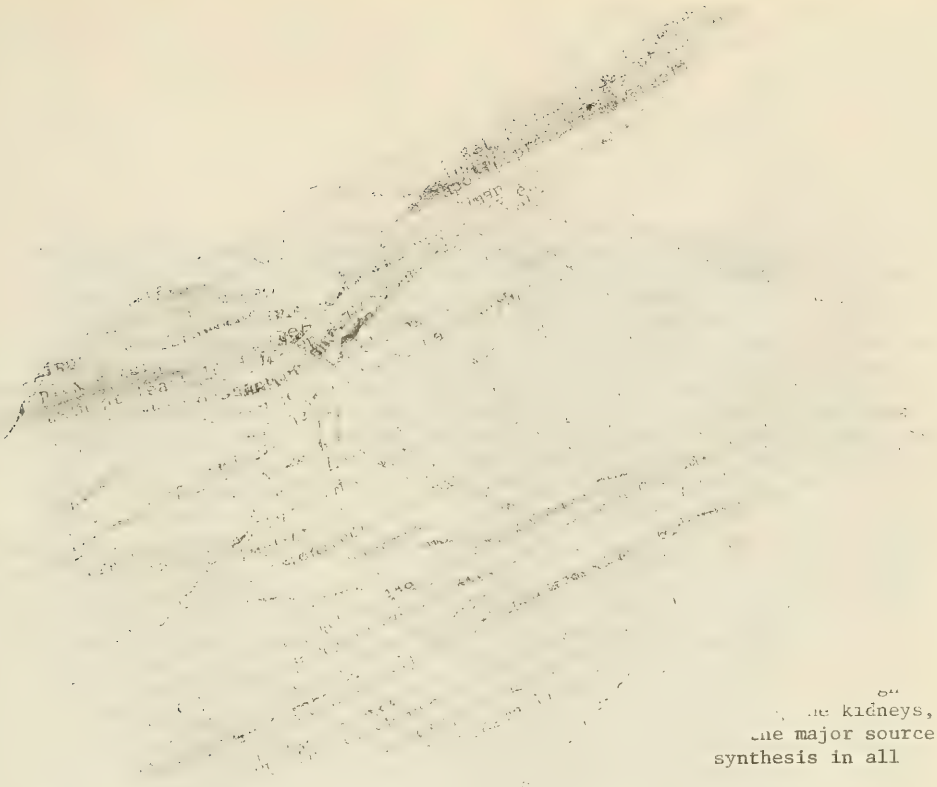
Nutritional Biochemistry Section

Role of the Small Intestine in Whole Body Nitrogen Metabolism

Earlier work from our laboratory demonstrated that mucosal epithelial cells of the small intestine in most animals, including primates, are a major site for the metabolism of circulating glutamine, the most abundant amino acid in the blood and the main end-product of nitrogen metabolism in skeletal muscle. In the small intestine, oxidation of the glutamine carbon provides an important source of energy; in rats in the postabsorptive state, 35% of the respiratory CO_2 produced by the intestine was shown to be derived from the metabolism of glutamine. We have now determined the metabolic fate of the glutamine nitrogen atoms. Using an autoperfused preparation of rat jejunum developed in this laboratory, we have quantified the uptake rates of nitrogenous metabolites by intestine from arterial blood *in vivo* as well as the release rates of nitrogenous products into intestinal venous blood. The only nitrogen-containing metabolite extracted in large quantitities was glutamine. The amounts of glutamine nitrogen taken up were balanced by the rates at which nitrogen was released as ammonia (38%), citrulline (28%), alanine (24%) and proline (7%).

Additional studies were then performed to assess the quantitative importance of this nitrogen-processing function of the intestine. The ammonia, alanine, and proline released by intestine are transported via the portal vein directly to the liver where all three are readily utilized as nitrogenous precursors for urea biosynthesis. The rate of hepatic urea biosynthesis was determined by measuring the urinary output of urea in fasted as well as non-fasted rats. The results show that in fasted animals about 30% of the urea produced in the liver is synthesized from nitrogenous precursors derived from the metabolism of circulating glutamine in the small intestine. In non-fasted animals, the rate of circulating glutamine metabolism by intestine increases about 2-fold. In addition, the fed intestine metabolizes most of the dietary glutamine and all of the dietary aspartate and glutamate, yielding the same urea precursors derived from circulating glutamine. Our quantitative measurements reveal that in the fed animal 50-60% of hepatic urea synthesis utilizes nitrogenous substrates derived from the intestinal metabolism of dietary and circulating amino acids. Amino acids are clearly an important source of energy for the intestine; furthermore, the intestine is a site where the nitrogen derived from muscle and from certain dietary amino acids, toxic if absorbed in large quantities, is converted to forms utilized readily by the liver for conversion to urea, which is non-toxic and eliminated from the body by the kidneys.

Citrulline in the liver is an intermediate metabolite on the pathway leading to the biosynthesis of arginine and urea. From vascular perfusion



...e kidneys,
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...n-molecular weight complexes that serve
...ious lipids among the body tissues. Elevated
...of certain plasma lipoproteins and depressed levels of others are associated with abnormal deposition of lipids in arterial endothelial cells (arteriosclerosis). We still know relatively few details regarding lipoprotein structure, synthesis, and metabolism, owing largely to the complex nature of these heterogenous particles. Rat plasma lipoproteins contain at least 10 different polypeptides or apolipoproteins, largely analogous to the constituent apolipoproteins of human plasma. Physiological functions have been deduced for some of these proteins -- in the assembly and transcellular transport of these complexes and in their metabolism in blood and other tissues. The functions of some other of these proteins remains obscure.

In previous work from this and other laboratories, liver and intestine were identified as the only sources of plasma apolipoproteins. The pattern of hepatic apolipoprotein synthesis was determined in isolated, perfused rat livers. To determine the pattern of intestinal synthesis, we developed a preparation of autoperfused rat small intestine which permitted recovery of intestinal lymph, important because most intestinal lipoprotein products reach the blood via the lymph. Liver and intestine were found to have very different but overlapping patterns of apolipoprotein synthesis. Since several of the proteins were synthesized by both organs, two methods were developed to quantitate the relative contributions of each. In method 1, intestinal and hepatic products are differentially labeled by administering [^{3}H]leucine intraduodenally and [^{14}C]leucine intraportally to conscious rats.

The $^{3}H/^{14}C$ ratios of individual plasma apolipoproteins are determined and related mathematically to reference proteins from liver (albumin) and intestine (selected apolipoproteins isolated from mesenteric lymph). In method 2, the patterns of apolipoprotein synthesis observed in isolated liver and in isolated intestine are related by multiple regression analysis to the pattern observed in the intact rat, where the contributions of both organs are reflected. Results with the two complementary methods were in good agreement and, when applied in fat-fed rats, showed that intestine contributed 50-60% of two of the major proteins of plasma high-density lipoproteins, namely apolipoproteins A-I and A-IV (apoA-I and apoA-IV). About 20% of the circulating apoB (major protein of low density lipoprotein) was also synthesized by the intestine.

To determine if this unexpectedly large intestinal contribution of several apolipoproteins was related to the transport of dietary fat, the same methods have now been applied in two groups of rats not given fat. In one group, all dietary fat was withheld for 24 hours. In another group, a fat-free diet was supplied for 10-14 days, and in addition, hepatic bile was diverted from the intestine to also eliminate the major endogenous source of lipids in the intestine. The results indicate that while the absence of fat reduces the intestinal production of apoB, intestinal production of apoA-I and apoA-IV continue at about the same rates as during massive fat transport. The route by which these proteins enter the circulation is quantitatively altered, however. During fat transport, most of the newly-synthesized intestinal protein enters via mesenteric lymph in association with chylomicrons, large triacylglycerol-rich lipoprotein particles. In the absence of dietary or endogenous luminal lipid, more of these proteins enter intestinal venous blood directly, by-passing the lymph.

Preliminary results have also been obtained in rats made hypercholesterolemic by additions of cholesterol, taurocholate and propylthiouracil to the diet. In these animals, compared with normally fed controls, the rates of apoB synthesis relative to the other apolipoproteins appear to be increased. The increase appears due to a change in the pattern of apolipoprotein synthesis in the liver. (A.-L. Wu and H. Windmueller)

Metabolism of Fat-Soluble Vitamins

We observed that rats fed two different forms of vitamin A - retinol and retinoic acid - had markedly different plasma levels of α-tocopherol (vitamin E). Retinol is the normal dietary form of vitamin A, whereas retinoic acid is considered to be a normal metabolite that supports all biochemical functions except those of vision and reproduction. Weanling rats were fed a complete purified diet with either retinol or retinoic acid at 4 mg/kg diet. This is a low, normal amount of vitamin A. After 2-4 weeks the rats were bled and their plasmas analyzed for retinol and α-tocopherol simultaneously by a high pressure liquid chromatography procedure developed in this laboratory. In as short a time as two weeks, feeding retinoic acid resulted in a 50% reduction of plasma α-tocopherol when compared to rats fed retinol. Analyses of liver and adipose tissue showed a similar reduction. This effect of retinoic acid was also demonstrated in chicks. Studies to elaborate

the mechanism of this vitamin interaction showed that retinoic acid did not accelerate the loss or destruction of endogenous α-tocopherol. Using ^{3}H-α-tocopherol, it was found in both rats and chicks that the apparent intestinal absorption of α-tocopherol is impaired when retinoic acid is in the diet. In chicks fed retinoic acid, the percent of dose appearing in the blood 2.5 hours after oral administration of labeled α-tocopherol was only 22% that of chicks fed retinol. In rats with a mesenteric lymph cannula, the percent of dose recovered 6 hours after intraduodenal administration of labeled α-tocopherol was reduced by one-third by feeding retinoic acid. Analyses by thin layer chromatography of the recovered radioactivity revealed slightly more oxidative destruction of α-tocopherol in rats fed retinoic acid, but the difference was not enough to account for the magnitude of the difference in plasma α-tocopherol concentration. Analyses revealed that there were no differences in plasma triglycerides, cholesterol or total lipids in rats fed retinol vs. retinoic acid. (Other investigators have shown that higher dietary levels of retinoic acid cause an elevation of plasma triglycerides). Examination of the intestinal mucosa by electron microscopy revealed no morphological differences due to feeding retinoic acid (Dr. W.G. Robison, Jr., NEI). The mechanism by which low intakes of retinoic acid interfere with α-tocopherol absorption is being investigated. (J. Bieri)

The utilization of vitamins A and E in total parenteral nutrition in rats was investigated. A surgical technique for feeding rats totally by vein was developed for determining the maintenance of plasma and tissue levels of these two vitamins (with the advice of Dr. Martin Popp, NCI). It was found initially that these two fat-soluble vitamins, when dispersed in the aqueous parenteral nutrition solutions customarily used clinically, were almost totally adsorbed by the plastic tubing used for intravenous infusion delivery. Studies using model systems have shown that by elevating the vitamin concentration five to ten fold, saturation of the tubing occurs after 2-4 hours and the vitamins then are delivered at a constant rate to the animal. Experiments are currently underway to determine the tissue retention of vitamins A and E given parenterally for two weeks or longer. (M. McKenna)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15000-10 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanism of Action of Hormones and Guanine Nucleotides on Adenylate Cyclase Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Martin Rodbell	Chief	LNE	NIAMDD
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Others:	T.B. Nielsen	Staff Fellow	LNE	NIAMDD
	Dermot M.F. Cooper	Visiting Fellow	LNE	NIAMDD
	Michael C. Lin	Research Chemist	LNE	NIAMDD
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	M. Sue Preston	Biologist	LNE	NIAMDD

COOPERATING UNITS (if any)
Ellis Kempner, LPB, NIAMDD

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	2.8	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

As judged by guanine nucleotide exchange, the multi-receptor (R) system in the turkey erythrocyte (TE) plasma membrane shows that adenosine has access to fewer regulatory components (N), at a slower rate than does isoproterenol. Adenosine inhibition is mediated through the P site by a process not involving nucleotide exchange. By the use of a complementation assay it was shown that the TE system can function adequately as an acceptor for N. Irradiation analysis of the TE system shows that it consists of multi-subunit form of R and a multi-subunit form of N, linked to the catalytic unit in a precoupled complex in the plasma membrane.

Project Description

Objectives: One of the long standing objectives of the laboratory has been the understanding of the structure and function of the adenylate cyclase system. Although most systems consist of the same three regulatory units (the hormone receptor R, the nucleotide regulatory component N, and the catalytic unit C), several functional variations in the regulatory properties exist from system to system. We have chosen to study the turkey erythrocyte system because it displays two such closely related and important functional differences. 1) The nucleotide regulatory component binds the inhibitor GDP tightly: thus Gpp(NH)p, a good activator in most cyclase systems, activates poorly in the turkey erythrocyte system. 2) Activation by hormone + GTP is appreciably lower than activation by either fluoride or hormone + Gpp(NH)p.

The multi receptor hormone system was analysed by examining in detail the pattern of removal of GDP by two distinct agents acting through separate receptors, adenosine and isoproterenol. We have used one aspect of the functional inertness of the system to Gpp(NH)p to develop a simple complementation assay for the N unit which in comparison with other assays can be used to assess the organization of the catalytic unit in the plasma membrane. Finally we have analyzed the structure of the system by irradiation analysis. Comparison of the structures of this system as opposed to a functionally complete system such as the liver adenylate cyclase system provides important clues about the organization of adenylate cyclase systems in general.

Methods Employed: The preparation of the turkey erythrocyte and human erythrocyte membranes, the procedures for irradiation analysis and guanine nucleotide exchange have been described in detail in last year's report.

Major Findings: The access of two distinct hormone-receptor complexes to the receptor regulatory component (N), was assessed by pretreatment of the membranes with either agent and GMP, followed by washing to remove these agents, and subsequent assay for Gpp(NH)p and isoproterenol + Gpp(NH)p activities. The ratio of these activities has been used as a measure of exchange. It was found that adenosine acting through its receptor has access to fewer N units, at a slower rate, than does isoproterenol. It is not yet apparent whether this pattern of exchange arises from distinct RN complexes existing within the membrane or whether it reflects the slow exchange of the adenosine receptor (uncoupled to N) with a beta receptor coupled to the N unit. It is abundantly clear however that the extent and rate of access of receptors, to their regulators, can be very different in a single plasma membrane.

Apart from the process of activation, adenosine inhibits cyclase through the P site. This site can be easily distinguished by the use of analogs such as dideoxyadenosine. It was shown that the pattern of inhibition of adenosine through the P site is very different from the pattern of activation by adenosine through the receptor. The most important difference is that the P site does not participate in guanine nucleotide exchange whereas the adenosine receptor clearly does. Thus in a functional sense the P site is located distal to the nucleotide component but is proximal to the catalytic unit.

The functional organization of the turkey erythrocyte system was probed by the use of a complementation assay. Addition of the human erythrocyte membrane (which contains N but no R or C) to the turkey erythrocyte membrane (R, N-GDP,C) causes an increase in activation by Gpp(NH)p activation. This indicates that the C unit in the turkey erythrocyte membrane is available for functional linkage with exogenously added N. That this linkage occurs through the N component in the human erythrocyte is easily confirmed by treatment of the human erythrocyte membrane with cholera toxin and NAD prior to complementation. Such pretreatment causes an increase in GTP activation of the system. Comparison of the extent of this complementation with the complementation of N to an N^- membrane indicates that the former is less efficient with the turkey erythrocyte as acceptor. Thus, while C can be multivalent it is only reluctantly so when it is already precoupled to an N unit in the plasma membrane. When complementation is carried out in the presence of endogenous activators (fluoride, isoproterenol + GTP, or isoproterenol + Gpp(NH)p) no further activation results upon the addition of exogenous N. Two results follow from this experiment. a) The functional stoichiometry of the turkey erythrocyte system is $RN \geq C$. b) As the isoproterenol + GTP activation is not enhanced, the process of exchange is not influenced by the addition of N. Thus the structural alterations which give rise to altered exchange and lower hormone + GTP activation cannot arise merely from a deficiency of N units.

Irradiation analysis of the turkey erythrocyte system revealed that increasing functional complexity is reflected in increasing structural complexity in this system. Thus the target size of MnATP turnover (C) has a target size of 100,000. The size requirement for fluoride or Gpp(NH)p stimulated activites (NC) is 230,000. As the subunit size of the N unit as determined by toxin labeling is 40,000 it follows that the N unit exists in the membrane as a multisubunit protein. The target size for hormone + Gpp(NH)p activity (RNC) is 320,000. Thus the structural contribution of the receptor is 90,000. This is directly verified by determining the target size for antagonist binding ($I^{125}I_{HYP}$) to the beta receptor. This target size is also 90,000. As the subunit size of the beta receptor is thought to be 36,000 to 39,000, the beta receptor is also a multisubunit protein.

The following important conclusions emerge from these findings.

1. The turkey erythrocyte system consists of precoupled RN, NC complexes. As we have shown in last year's report, this is in complete agreement with the fact that after nucleotide exchange to remove the inhibitor GDP, nucleotide regulation of the receptor as well as the catalytic unit can be easily detected.

2. In contrast to the liver adenylate cyclase system the turkey erythrocyte system is missing the large oligomeric complexes which have been observed in the liver cyclase system. As to whether this absence is indicative of the obligatory role of these complexes in hormone + GTP activation has still to be established. However as it remains the only significant difference in structure between these two systems (the size requirements for NC and C mediated activities are very similar); we must assume that the absence of these structures is a significant and perhaps causative element contributing to the functional differences.

3. Removal of GDP from the N unit does not cause any change in the structure of the turkey erythrocyte system. Thus GDP binding is not the causative event in the occurrence of this structure. Rather some other structural change either causing or accompanying the change to tight GDP binding seems to be indicated.

4. Finally the multi subunit organization of the receptor as well as its regulator renders comprehensible early observations in the literature. These include differences in agonist vs. antagonist binding, the requirement for GMP in exchange, the apparent two site type of nucleotide reactivity, and the existence after solubilization of separable RN, NC complexes.

Significance to Biomedical Research and the Program of the Institute: Receptor malfunction can be involved in a number of disease related states. Detailed knowledge of the structure and function of receptors and receptor regulators in the plasma membrane would be useful at least in the typing of various disease states if not immediately responsible for a cure. The studies described here outline a biochemical and biophysical approach that could be very useful in this general endeavor.

Proposed Course of Project: Terminated.

Publications

Rodbell, M.: The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature 284, 17-22, 1980.

Lad, P.M., Nielsen, T.B., Preston, M.S. and Rodbell, M.: The role of the guanine nucleotide exchange reactions in the regulation of the β-receptor and in the actions of cholera toxin on turkey erythrocyte adenylate cyclase. J. Biol. Chem. 255, 988-995, 1980.

Nielsen, T.B., Lad, P.M., Preston, M.S. and Rodbell, M.: Characteristics of the guanine nucleotide regulatory component of adenylate cyclase in human erythrocyte membranes. Biochimica et Biophysica Acta 269, 143-155, 1980.

Lad, P.M., Nielsen, T.B., Londos, C., Preston, M. Sue and Rodbell, M.: Adenosine activates and inhibits turkey erythrocyte adenylate cyclase by distinct processes. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15001-06 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure-function Relationship of Glucagon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Martin Rodbell	Chief	LNE	NIAMDD
	David E. Wright	Staff Fellow	LNE	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A sulfhydryl group was introduced into the glucagon molecule by reacting glucagon with 2,4 dinitrophenylsulfenyl chloride. The resultant Trp-(2-dinitrophenylsulfenyl)-glucagon was reduced by thiolytic cleavage to 2-thiol-Trp-glucagon. The thiol derivative oxidizes to the dimer $(Trp\text{-}S\text{-}glucagon)_2$ at pH 9.0 when exposed to air. All three thiol derivatives of glucagon proved to be equipotent on a molar basis with glucagon in the activation of adenylate cyclase in rat liver plasma membranes. These findings indicate that bulky constituents on the Tryptophan residue do not interfere with binding and action under assay conditions. However, it was found that the glucagon dimer binds to the glucagon receptor with lower affinity than the glucagon monomer. GTP, which modifies the glucagon receptor and which activates adenylate cyclase through a GTP-regulatory unit causes the receptor to bind equally the dimeric and monomeric forms of glucagon. These findings provide supportive evidence that the glucagon receptor exists in an oligomeric structure in which, perhaps because of steric hindrance, the bulky glucagon appendage on the dimer interferes with binding. GTP, by dispersing the oligomeric structure to monomers, allows the dimer to bind (and act) with potency equivalent to that of glucagon.

PHS-6040

Project Description

Objectives: A major goal of this project is to determine the structural requirement for glucagon binding and action at its receptor. Such knowledge subserves several purposes, including "mapping" the domains or regions of the receptor that complement the structure of the hormone, the possibility of synthesizing inhibitory derivatives of the hormone, and a means of attaching the hormone to its receptor for identification and purification purposes.

Methods Employed: Glucagon was reacted with 2,4 dinitrophenylsulfenyl chloride to form 2-trp-dinitrophenylsulfenyl-glucagon. This compound was reduced by thiolysis to form 2-thiol-Trp-glucagon. Air oxidation of 2-thiol Trp-glucagon at pH 9.0 was a means of forming a disulfide dimer of glucagon (Trp-S-glucagon)$_2$. Each derivative of glucagon was purified on ion exchange columns and identified by a variety of physical determinations. The compounds were tested for their biological activity and potency on adenylate cyclase activity in rat liver plasma membranes; their affinities for the glucagon receptor were determined from the ability to displace the binding of ^{125}I-glucagon to specific receptors in the liver membranes.

Major Findings: The dose-response relationship for activation of adenylate cyclase activity in liver membranes was identical to that of glucagon for each of the glucagon derivatives modified at the tryptophan residue. On a molar basis, the glucagon dimer was identical to that of native glucagon, indicating that the receptor recognizes only one of the glucagon molecules of the dimer. Thus, the bulky glucagon appendage attached to the thiol-Trp residue of glucagon did not interfere with the binding and action of glucagon. By contrast, the affinity of the glucagon dimer (again on a molar basis) for the glucagon receptor as measured under non-cyclase assay conditions (i.e., no GTP was present) was about 1/6 that of native glucagon. This reduction in affinity was not seen with 2,4 dinitrophenyl-glucagon which had the same affinity for the receptor as native gluagon. However, when GTP was added to the assay medium used for investigating binding, the dimeric form of glucagon demonstrated an equivalent affinity for the receptor as that of native glucagon. Thus, under conditions in which glucagon activates adenylate cyclase and in which GTP modifies the affinity of the receptor for the hormone, the glucagon dimer binds to the receptor as if it were native glucagon. These findings are consistent with our proposal, based on target analysis of the structure of the glucagon-sensitive cyclase system, that the glucagon receptor is present in the membrane as an oligomer of receptors linked to the GTP-regulatory units and that binding of hormone and GTP to their respective units depolymerizes the oligomers to smaller units (monomers of receptor-GTP regulatory unit) that are capable of coupling to adenylate cyclase and activating the enzyme. We interpret the findings with the glucagon dimer as evidence that the oligomeric form of the receptor is structured such that the glucagon appendage of the dimer interferes, possibly by steric hindrance, with the binding of glucagon. Dispersion of the oligomer by the actions of glucagon and GTP produces a complex in which the appendage is no longer capable of interfering with binding.

Significance to Biomedical Research and the Program of the Institute: Studies of the structure-function relationships in hormones are essential for understanding the mechanism of action of hormones at their receptors and for understanding the physical properties of these biologically important agents. Potentially such information can give insights for the chemical synthesis of new forms of the hormone that can be used for understanding the molecular basis of hormone action and for clinical purposes.

Proposed Course of Project: Terminated.

Publications:

Wright, D.E. and Rodbell, M.: Preparation of 2-thiol-Trp-glucagon (Trp-S-glucagon)$_2$: Differences in binding to the glucagon receptor in the hepatic adenylate cyclase system. J. Biol. Chem., in press, 1980.

Wright, D.E. and Rodbell, M.: Properties of amidinated glucagons. Eur. J. Biochem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15004-05 LNE

PERIOD COVERED
October 1, 1979 through September 31, 1980

TITLE OF PROJECT (80 characters or less)
Studies of Hormone Responses in Cultured Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Michael C. Lin	Research Chemist	LNE NIAMDD
Other:	Shay-Whey Margaret Koh	Visiting Fellow	LNE NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An established kidney cell line (MDCK) contains an adenylate cyclase system sensitive to the effects of glucagon, vasopressin, isoproterenol and prostaglandin E_1. We have found that the effects of the peptide hormones were selectively lost or reduced in a cloned line derived from MDCK cells transformed by Harvey murine sarcoma virus. Isoproterenol and prostaglandin E_1 responses were unchanged. Glucagon sensitivity can be restored by including butyrate in the culture medium or by prolonged culture in a chemically-defined medium containing hormone supplements. Disappearance and reappearance of glucagon receptors seem to be responsible for the loss and restoration of glucagon sensitivity. The study of this system will provide an understanding of the cellular regulation of hormone receptors, their response and the biochemical pathway leading to the appearance of hormone receptors.

Project Description

Objectives: The goals of this project are: 1) To study the factors in the cellular environment required for maintaining the hormone responsiveness, as evidenced by hormone sensitivity of adenylate cyclase system; and 2) To examine the biochemical basis for the loss and reappearance of glucagon response in an established kidney cell line (MDCK).

Methods Employed: The kidney cell line (MDCK) and a clone of virally transformed MDCK cells were maintained as monolayer culture in Dulbecco MEM with 10% fetal bovine serum or with a hormone supplement. Effects of hormones were determined 1) by measuring the intracellular cyclic AMP by a radioimmunoassay, 2) by assaying for adenylate cyclase activity in the isolated plasma membranes by procedures established in this laboratory and 3) by measuring the binding of radioactively labelled hormones to their receptors. Cell growth was quantitated with a cell counter.

Major Findings: MDCK cells contain an adenylate cyclase system that is sensitive to the stimulatory effects of glucagon, vasopressin, isoproterenol and prostaglandin E_1. We have found that the effects of the peptide hormones were selectively lost or reduced in a cloned line derived from MDCK cells transformed by Harvey murine sarcoma virus. Isoproterenol and prostaglandin E_1 responses were unchanged. This selectivity in altered hormone responsiveness suggests that each type of hormone response is under unique regulation. Glucagon sensitivity can be restored by including butyrate in the culture medium for 3 days or by culturing the cells in a chemically-defined medium containing hormone supplements for 7 days. The dose-response relationship for butyrate-induced glucagon response showed a reduced efficacy for glucagon. This aberration seems to be intrinsic to the viral transformation and specific for glucagon system. Disappearance and reappearance of glucagon receptors seem to be at least partly responsible for the loss and restoration of glucagon sensitivity. We have also found that tunicamycin, a glycosylation inhibitor, blocked the induction of glucagon response by butyrate suggesting that the glucagon receptor is a glycoprotein.

Significance to Biomedical Research and the Program of the Institute: Changes in hormone sensitivity are frequently observed during ontogenetic development and as a result of altered hormonal environment. Cells in culture are suitable for studying the factors involved in such changes since it is possible to systematically alter the cellular environment for differentiation and growth of the cells. Our initial study suggests that the hormone receptor is probably the site of regulation. Therefore this project will provide an understanding of the cellular regulation of hormone receptors, their response and the biochemical pathway leading to the appearance and disappearance of hormone receptors.

Proposed Course of Project: Our current and future efforts include: 1) Elucidating the cause for the shift in the dose-response relationship for glucagon in the butyrate-treated transformed cells by examining the characteristics of the isolated plasma membranes; 2) Establishing the growth require-

of the transformed MDCK cells when cultured in a defined medium; 3) Determining the factors responsible for the induction of glucagon sensitivity when transformed cells are cultured in the defined medium; and 4) Studying the biosynthetic pathway leading to the appearance of glucagon receptors.

Publications

Lin, C-S. and Lin, M.C.: Appearance of β-adrenergic response of adenylate cyclase during the induction of differentiation in cell cultures. Exp. Cell Res. 122: 399-402, 1979.

Cooper, D.M.F., Schlegel, W., Lin, M.C. and Rodbell, M.: The fat cell adenylate cyclase system. Characterization and manipulation of its bimodal regulation by GTP. J. Biol. Chem. 254: 8927-8931, 1979.

Moss, J., Stanley, S.J. and Lin, M.C.: NADglycohydrolase and ADP-ribosyltransferase activities are intrinsic to the A_1 peptide of choleragen. J. Biol. Chem. 254: 11993-11996, 1979.

Lin, M.C.: Long term maintenance of hormone responsiveness in primary hepatocyte culture. Ann. N.Y. Acad. Sci. in press, 1980.

Lad, P.M., Nielsen, T.B., Lin, M.C., Cooper, D.M.F., Preston, M.S. and Rodbell, M.: Toward a unifying hypothesis for the effects of cholera-toxin-catalyzed ADP-ribosylation in diverse adenylate cyclase systems. In Sugimura, T. and Smulson, M. (Ed.): Novel ADP-ribosylation of regulatory enzymes and proteins, New York, Elsevier-North Holland Press, 1980, pp. 1-35.

Lin, M.C., Cooper, D.M.F. and Rodbell, M.: Selective effects of organic mercurials on the GTP-regulatory proteins of adenylate cyclase systems. J. Biol. Chem. in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15005-05 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Actions of Adenosine on Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	D.M.F. Cooper	Visiting Fellow	LNE	NIAMDD

COOPERATING UNITS (if any)
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Laboratory of Nutrition and Endocrinology

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Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	none

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A. We have established conditions for demonstrating that antilipolytic agents, such as prostaglandins, nicotinic acid, and adenosine, inhibit adenylate cyclase in purified fat cell plasma membranes. The antilipolytics inhibit activity by acting at receptors identical to those through which they inhibit lipolysis in intact cells. Moreover, as with their physiological effects, antilipolytics act only against low activity states of the enzyme. Thus, by both kinetic and pharmacological criteria, these findings indicate taht antilipolytic agents exert their effects by acting directly on the adipocyte adenylate cyclase.

B. Examination of adenylate cyclases from different cells strengthens our previous conclusion that adenosine receptors differ both functionally and pharmacologically. Studies with the turkey erythrocyte adenylate cyclase show that the adenosine receptor, like other hormone receptors, acts by releasing bound inhibitory GDP from the GTP-binding regulatory component of the cyclase system.

Project Description

Objectives: A) Previously we established conditions for demonstrating that receptors for the potent antilipolytic agent, adenosine, were linked to and promoted inhibition of the fat cell adenylate cyclase in purified plasma membranes. We then asked whether other antilipolytic agents, such as prostaglandins and nicotinic acid, also inhibit the adipocyte cyclase, and, if so, whether they exerted their effects via mechanisms similar to those found for adenosine receptors.

B) Based on studies with adenylate cyclase from 3 different cells we had proposed that distinct subclasses of adenosine receptors could be distinguished by functional and pharmacological criteria. In order to determine whether this classification applied generally, we examined other cells known to contain stimulatory receptors and conducted a search for cells containing inhibitory adenosine receptors. Finally, we examined in detail the turkey erythrocyte adenylate cyclase in order to determine if adenosine receptors share mechanisms with other hormone receptors in their stimulation of adenylate cyclase.

Methods Employed: An adenylate cyclase assay method was developed in which deoxy ATP (dATP) was employed as substrate. We had found in previous studies that deoxyadenosine is a poor effector at adenosine receptors; thus production of deoxyadenosine from dATP should not interfere with the actions of adenosine or other analogs added to the medium. The assay previously developed in this laboratory for measuring cyclic AMP had to be modified for the assay of deoxycyclic AMP produced from labeled dATP. In all other respects, the studies reported were carried out with techniques established in this laboratory.

Major Findings: A) Studies on the effects of antilipolytic agents on fat cell adenylate cyclase in purified plasma membranes reveal the following:

1. Like adenosine, prostaglandins and nicotinic acid inhibit activity;
2. These three agents act by binding to different receptors;
3. Inhibition by all three agents is mediated by a GTP-dependent process different from the GTP-dependent process that mediates adenylate cyclase stimulation by lipolytic hormones (catecholamines, glucagon, ACTH, etc.);
4. Sodium ions enhance the inhibition by antilipolytic agents by reducing the inhibitory action of GTP seen in the absence of these agents.

The following findings reveal striking parallels between the actions of antilipolytics on adenylate cyclase in purified membranes and their effects on the lipolytic process in intact adipocytes.

1. The receptors for prostaglandins, adenosine, and nicotinic acid that mediate inhibition of adenylate cyclase are pharmacologically identical to the receptors through which these agents exert their effects on lipolysis in intact adipocytes;

2. Inhibition is limited to low activity states of the enzyme, but independent of the lipolytic hormone used to stimulate activity;

3. Each antilipolytic agent produces partial inhibition of cyclase, but combinations of antilipolytics produce no greater inhibition than does the most effective agent when tested alone. This final point reveals that the effectiveness of antilipolytics is limited by factors other than receptors numbers. It seems likely that the nucleotide inhibitory process governs the effectiveness of antilipolytic agents.

B) As with adenylate cyclase in membranes from Leydig tumor cells and liver, adenosine receptor-mediated stimulation of cyclases from Y-1 adrenal cells and turkey erythrocytes requires lower concentration of 5'-N-ethylcarboxamineadenosine (NECA) than N^6-phenylisopropyladenosine (PIA). In contrast to the above enzymes, but similar to the fat cell cyclase, inhibition of brain cortical cyclase is more sensitized to PIA than NECA. Thus, it would appear that our previous subclassification of adenosine receptors, based on functional (stimulation versus inhibition) and pharmacological criteria (NECA versus PIA) apply generally.

It is known that agonists of the beta-adrenergic receptor in turkey erythrocyte membranes stimulate adenylate cyclase by causing release of GDP bound to the cyclase-associated guanine nucleotide regulatory component. We now find that agonists of adenosine receptors in these same membranes stimulate activity by an identical mechanism. This extends our previous conclusions on the similarities between adenosine receptors and receptors for other hormones. Finally, we have determined that recent proposals on the unique relationship between adenylate cyclase and adenosine receptors ("tightly-coupled" receptors) are based on artifacts.

Significance to Biomedical Research and the Program of the Institute: Since the discovery that a substantial fraction of food energy is cycled through fat cells, considerable effort has been expended in an attempt to elucidate the mechanisms by which lipids are stored and mobilized. It is generally accepted that lipolytic hormones mobilize lipids by increasing cellular cyclic AMP concentrations, and these hormones stimulate adenylate cyclase directly. Our studies show, for the first time, that various antilipolytic hormones act also on the cyclase system in purified membranes. Thus, one can now examine the rate-limiting steps for hormonal regulation of lipolysis with a single enzymatic reaction in purified membranes. Perhaps even more important than their implications for control of lipolysis, these findings reveal the dualistic nature of adenylate cyclase regulation by hormone receptors. It is likely that our conclusions on the regulation of the adipocyte enzyme will hold generally for most cyclases, albeit with different classes of stimulatory and inhibitory hormones.

Adenosine acts at cell surface receptors to mediate a wide variety of biologic effects, such as coronary artery dilation, suppression of immune function in lymphocytes, and potentiation of histamine release in mast cells. Considerable effort is now being put forth in the search for pharmacologically active agonists and antagonists of the adenosine receptor(s). Our studies reveal that there are distinct subclasses of adenosine receptors. Moreover, the methods we have developed provide a means for rapid testing and screening of pharmacologic agents.

Proposed Course of the Project: Further studies will involve: 1) biochemical identification of adenosine recpetors with the use of radiolabeled photoaffinity adenosine analogs; and 2) with the use of knowledge gained from our studies on the inhibitory effect of adenosine on the fat cell cyclase, an attempt will be made to resolve the controversial question as the actions of insulin on adenylate cyclase. 3) In an attempt to approach the biochemical basis for the limited ability of antilipolytic agents to inhibit both lipolysis and adenylate cyclase, we will investigate the effects of altered thyroid status on the balance between the actions of lipolytic and antilipolytic agents on adenylate cyclase in fat cell membranes. Recent publications suggest that fat cells from hypothyroid animals exhibit a poor lipolytic response to catecholamines not, as had been thought previously, because they contain fewer adrenergic receptors, but because of enhanced efficacy of the adenosine response.

Publications

Londos, C., Wolff, J. and Cooper, D.M.F.: Action of adenosine on adenylate cyclase. In: Baer, H.P. and Drummond, G.I. (Eds.): Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides. New York, Raven Press, 1979, pp. 271-281.

Cooper, D.M.F. and Londos, C.: Evaluation of the effects of adenosine on hepatic and adipocyte adenylate cyclase under conditions where adenosine is not generated endogenously. J. Cyclic Nucleotide Res. 5, 289-302, 1979.

Londos, C., Cooper, D.M.F. and Wolff, J.: Distinct Subclasses of adenosine receptors. Proc. Natl. Acad. Sci. USA 77, 2551-2554, 1980.

Londos, C., Wolff, J. and Cooper, D.M.F.: Adenosine regulation of adenylate cyclase. In: Burnstock, G. (Ed.): Purinergic Receptors. London, Chapman and Hall, in press.

Cooper, D.M.F., Londos, C. and Rodbell, M.: Adenosine receptor-mediated inhibition of rat cerebral cortical adenylate cyclase. Mol. Pharmacol. in press.

Londos, C. and Cooper, D.M.F.: Stimulation and inhibition of adenylate cyclases by hormones: The adipocyte as a model system. In: Robison, G.A., Greengard, P. and Dumont, J. (Eds.): Adv. Cyclic Nucleotide Res. 14. New York, Raven Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15006-05

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of the GTP-regulatory Component of Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: T.B. Nielsen Staff Fellow LNE NIAMDD

COOPERATING UNITS (if any)

A. Spiegel, R. Downs, G. Aurbach NIAMDD

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

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INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated.

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15007-02 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Target Size Analysis of Adenylate Cyclase and Hormone Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Werner Schlegel	Visiting Fellow	LNE	NIAMDD
Martin Rodbell	Chief	LNE	NIAMDD

COOPERATING UNITS (if any)

E.S. Kempner LPB, NIAMDD

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NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 15010-02 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

GTP-mediated stimulation and inhibition of adenylate cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	Constantine Londos	Research Chemist	LNE	NIAMDD
	Werner Schlegel	Visiting Fellow	LNE	NIAMDD
	Martin Rodbell	Chief	LNE	NIAMDD

COOPERATING UNITS (if any)

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Laboratory of Nutrition and Endocrinology

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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2.5		

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Examination of adenylate cyclase in plasma membranes preparations from rat adipocytes, brain cortex, cordate nucleus and human platelets revealed that activity is inhibition by putative neurotransmitters. Depending on the cell from which membranes were derived, inhibition was seen with adenosine, epinephrine, prostaglandins and opiates. As with stimulation of cyclases by hormones and neurotransmitters, inhibition was dependent on GTP. However, in contrast to stimulatory hormones, inhibition was: 1) enhanced by sodium and lithium ions 2) not expressed in the presence of Gpp(NH)p. Moreover, chemical treatments of membranes selectively destroyed the effects of stimulatory hormones, but not of inhibitory hormones, and vise versa. Finally, target size analysis indicated that the GTP dependent stimulatory and inhibitory actions are mediated by structures of different size. It is concluded that dual regulation (stimulation and inhibition) by receptor-mediated processes is a widespread feature of adenylate cyclase.

Project Description

Objectives: Previously we have demonstrated that adenylate cyclase activity of fat cell plasma membrane is subject to dual regulation by GTP-stimulatory phase which mediates that actions of stimulatory hormones and an inhibitory phase which promotes inhibition by adenosine and its analogs. We have accumulated evidence which suggests that the proteins mediating the two processes are distinct and have suggested that the fat cell system may be a model whereby other such systems can be evaluated.

In the present study we have used our experience of the fat cell to i) examine and discover a number of other systems in which such dualistic regulation operates ii) to determine whether features are shared by these systems in order to make their detection more predictable and to speculate on their minimal structural complexity iii) to demonstrate regulatory effects of neurotransmitters which had hitherto been undetected and iv) to probe with the technique of target size analysis structural aspects of the fat cell system.

Methods Employed: Plasma membranes were prepared by methods developed in this laboratory. Adenylate cyclase activity was assayed using either ATP, deoxy ATP or APP(NH)P as substrate with the elution modifications for the cyclic nucleotide which we have described. We developed the deoxyATP method in order to eliminate the generation of endogenous adenosine during the assay because of its potential as a potent inhibitor in these systems.

Major Findings: Using deoxyATP as substrate (and thus eliminating background adenosine) we have been able to detect inhibition by PGE_1 and nicotinic acid of the fat cell adenylate cyclase which is analogous to that mediated by adenosine. Combinations of maximally effective concentrations of inhibitors do not produce additive effects. This underlines the requirement for awareness of possible generation of endogenous inhibitors.

Our studies with brain cortical adenylat cyclase revealed the presence of dual regulation by GTP. Inhibition by GTP was promoted by adenosine with very similar pharmacological characteristics to that observed in the fat cell. Sodium ions amplified the effect.

Examination of caudate nucleus adenylate cyclase also revealed dualistic GTP regulation. Inhibition in this case was promoted by opiates such as morphine and enkephalins. This is the first demonstration of an opiate effect in an animal tissue preparation of adenylate cyclase. Amplification of the inhibitory effects was promoted by sodium ions.

In human platelet epinephrine mediates an alpha-2 adrenergic receptor inhibition of adenylate cyclase. This effect is absolutely dependent on the presence of a GTP-mediated inhibitory process, although in this case, there is no amplification of the effect by sodium.

In all cases, the non-hydrolysable GTP analog, Gpp(NH)p, would not substitute for GTP in promoting inhibition.

Examination of the fat cell enzyme by the technique of target size analysis demonstrated that the proteins mediating the inhibitory process were larger than those governing the stimulatory process. This represents further evidence for the distinctness of the two processes.

Significance to Biomedical Research and the Program of the Institute: The insights which we have gained on dualistic GTP regulation has already provided an in vitro capability for assessing responsiveness of target tissues to putative neurotransmitters. Future activity in this area will undoubtedly uncover effects of many other putative neurotransmitters at the level of adenylate cyclase, and this in turn may lead to a greater understanding of the physiological interaction between such regulators.

Proposed Course of Project: i) it is proposed to study interactions between stimulatory and inhibitory hormones/neurotransmitters by binding studies, as this may provide a means of identifying stimulatory/inhibitory circuits-other than by cyclase measurements. ii) Cholera toxin labelling will be attempted in order to determine whether the inhibitory protein can be monitored independently of its function (as the stimulatory GTP protein already has been so labeled) iii) A cell line has been identified which appears to contain the inhibitory GTP regulatory protein, although it does not appear to contain inhibitory receptors-this cell will be used as a potential source for the purification of the inhibitory GTP protein; these cells can also be studied with a view to inducing the appearance of inhibitory receptors; additionally, complementation studies will be attempted with inhibitory receptors from other tissues, in order to further our understanding on the assembly and constitution of such regulatory complexes.

Publications

Cooper, D.M.F., Schlegel, W., Lin, M.C. and Rodbell, M.: The fat cell adenylate cyclase system: Characterization and manipulation of its bimodal regulation by GTP. J. Biol. Chem. 254, 8927-8931. 1979.

Cooper, D.M.F. and Rodbell, M.: ADP is a potent inhibitor of human platelet plasma membrane adenylate cyclase. Nature 282, 517-518, 1979.

Cooper, D.M.F. and Londos, C.: Evaluation of the effects of adenosine on hepatic and adipocyte adenylate cyclase under conditions where adenosine is not generated endogenously. J. Cyc. Nucl. Res. 5, 289-302, 1979.

Schlegel, W., Cooper, D.M.F. and Rodbell, M.: Inhibition and activation of fat cell adenylate cyclase by GTP is mediated by structures of different size. Arch. Biochem. Biophys. 201, 678-682, 1980.

Londos, C., Cooper, D.M.F. and Wolff, J.: Subclasses of external adenosine receptors. Proc. Natl. Acad. Sci. 77, 2551-2554, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15011-02 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of Human Platelet Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Dermot M.F. Cooper	Visiting Fellow	LNE NIAMDD
	Martin Rodbell	Chief	LNE NIAMDD

COOPERATING UNITS (if any)
None

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated.

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15100-10 LNE

PERIOD COVERED
1 October 1979 to 30 September 1980

TITLE OF PROJECT (80 characters or less)

Protein-Nucleic Acid Interactions: Chromatin Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert T. Simpson	Medical Director (R)(T)	LNE NIAMDD
Others:	Peter Kunzler	Visiting Fellow	LNE NIAMDD
	Arnold Stein	Senior Staff Fellow	LNE NIAMDD

COOPERATING UNITS (if any)

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2.0	2.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

High precision membrane osmometry has demonstrated that histones in 2 M NaCl exist as a dissociating complex of octamers, hexamers and dimers; no evidence for the existence of a heterotypic tetramer is found. The stabilization of DNA in nucleosomes by interactions with histones is almost totally entropic in origin, consistent with the primary forces being electrostatic interactions. Under proper conditions, we demonstrate that H1 has two effects on reconstituted chromatin made with the four inner histones. First, it appears to alter the repeat length determined by micrococcal nuclease digestion from about 150 base pairs to over 180 base pairs, similar to native material. Second, it appears to alter the wrapping of DNA around the octamer such that the linking number change per nucleosome is altered from -1 to -2. NMR studies of 145 base pair poly(dG-dC) alone and in core particles show that the alternating conformation characteristic of the high salt form Z-DNA is not present when the polymer is associated with these basic proteins.

Project Description

Objectives: To study the structure of chromatin, the DNA-histone complex of eukaryotic cells, at various levels of organization. To define the mechanisms of packaging of DNA in the cell nucleus. To determine the features of chromatin structure which are associated with the function of the nucleoprotein in transcription, replication, and differentiation.

Methods Employed: Nuclei are prepared from HeLa cells, chicken erythrocytes or other tissues using non-ionic detergents. Nuclear suspensions in isotonic sucrose are digested with micrococcal nuclease and chromatin fragments (higher order chromatin to nucleosome core particles) isolated by sucrose gradient sedimentation. Synthetic polynucleotides are either purchased commercially, when available, or prepared by established methods. SV40 DNA form I is purchased; form I^r is prepared using topoisomerase I.

Histones are isolated from chromatin by either salt extraction or extraction with mineral acids. H1 (or H5) are isolated from total histones by selective extraction with 5% perchloric acid or by gel filtration in 2.5 M NaCl. Histones are associated with DNA by either salt step dialysis (2.5 M to 0.1 M) or by direct mixing in 0.1 M ionic strength in the presence or absence of polyglutamic acid.

Determinations of circular dichroism spectra, thermal denaturation profiles, sedimentation coefficients, ultracentrifugal equilibrium molecular weights, number average molecular weights by membrane osmometry, calorimetric heats, ^{31}P NMR spectra, DNA sizes and histone contents were made by established methods. Torsional constraints induced on SV40 DNA by association with histones were analyzed by agarose gel electrophoresis after relaxation with topoisomerase I and deproteinization by phenol extraction.

Major Findings: Although the inner histones are known to exist as a stable octamer in chromatin, the exact state of their association in the absence of DNA, but in high (2 M) salt solutions, has remained problematical. This is of interest, since several reports have suggested that the inner histones formed a heterotypic tetramer, one each of H2A, H2B, H3 and H4, and have attempted to attribute physiological significance to this species. We have now reexamined the state of aggregation of the inner histones using a method which obviates many of the experimental difficulties encountered by others.

We have measured the concentration dependence of the osmotic pressure for solutions of core histones in 2.0 M NaCl, pH 8.0, over the concentration range from 1 to 20 mg/ml at 25°C. The ratio of the osmotic pressure to the protein concentration is nearly constant over this range, with a value corresponding to an apparent number average molecular weight of 75,000. Gel filtration of a typical core histone preparation at 5°C at an initial concentration of 4.6 mg/ml resulted in the elution of 10% of the sample by weight as low molecular weight material consisting only of histones H2A + H2B, likely a dimer complex; 90% of the sample eluted at a volume close to that for a cross-linked histone octamer marker. The presence of this amount of low molecular weight material in the sample requires that the remaining material have a number average molecu-

lar weight of at least 92,500 to yield a measured value of Mn, over the whole sample, of 75,000. The existence of an appreciable amount of histone tetramers is therefore excluded for core histone concentrations greater than about 2 mg/ml. M_n for the purified high molecular weight fraction, however, was only 80,000, indicating that histones H2A + H2B interact with a larger complex in an association equilibrium. At 35°C, the concentration dependence of the osmotic pressure clearly indicated that an association equilibrium occurred. We show that the concentration dependence of the osmotic pressure for a solution of total core histones at 25°C can be described well by a dimer + hexamer $\rightleftharpoons$ octamer equilibrium with $K = (1.5 \pm 0.5) \times 10^{-2} M^{-1}$ and a second osmotic viral coefficient, $B=(1.8 \pm 0.17) \times 10^{-4}$ cm^3 mol g^{-2}, where the dimer and hexamer have the compositions (H2A H2B) and $(H3\ H4)_2$ (H2A H2B), respectively. These data, demonstrating that the heterotypic tetramer is not a stable constituent of histones in high salt concentrations in the absence of DNA, suggest reexamination of possible roles it might play in the cell.

We have also investigated the energic aspects of histone-DNA interactions using differential scanning calorimetry. Others have suggested a sizable enthalpic contribution to the stabilization of DNA structure by interactions with histones; based on theoretical considerations this would not be expected if the primary stabilizing forces were electrostatic in nature. Heats of thermal denaturation of chromatin core particles and core particles with covalently crosslinked histones were measured by differential scanning calorimetry. The additional stabilization of the nucleoprotein complex by crosslinking is not reflected in the transition enthalpy. The contribution of protein denaturation to the total heat was estimated by comparison of core particles with core particle DNA in a high salt solution. By taking into account the temperature dependence of the transition enthalpy of DNA, we conclude that the enthalpy change for denaturation of DNA in core particles is approximately the same as that for naked DNA in solution. The free energy of stabilization of nucleosomal DNA is about 66 kcal mol^{-1} greater than that for naked DNA, and may be primarily entropic in origin. This is consistent with the prime forces in DNA stabilization in chromatin arising from electrostatic interactions between the basic histones and the negatively charged phosphodiester backbone of the nucleic acid.

Beyond the core particle level of organization, chromosomal DNA is first packaged into the whole nucleosome, a structural repeating unit 20-100 base pairs longer than the core particle, and one which contains one molecule of the lysine-rich histone H1 (or H5 in chicken erythroctye) per nucleosome. We have investigated the role of H1 in generating this unit by in vitro association of histones and DNA. This problem is of particular importance since reconstitution of chromatin at levels higher than the core particle has never been achieved with fidelity and such reconstitution will be necessary for investigations of components sufficient and necessary for faithful transcription in vitro.

In agreement with others, addition of inner histones to DNA leads, after micrococcal nuclease digestion, to a series of fragments which are multiples of about 145 base pairs in length; these reflect the presence in the reconstituted chromatin of close-packed nucleosome core particles. Under proper con-

ditions, addition of H1 + H5, in the presence of polyglutamic acid, leads to a lengthening of the repeat to sizes over 180 base pairs. Optimization of conditions for attaining the longer repeat and investigation of different H1-equivalent molecules which are associated with different repeat lengths in vivo are currently underway.

H1 might also play a role in stabilizing wrapping of DNA around the core particle histone octamer; this was suggested by our demonstration that the chromatosome, a particle containing 160 base pairs of DNA and a molecule of H1, in addition to the octamer, had two full turns of DNA around the histone nucleus of the core particle. If nucleosomes associated with H1 were like the chromatosome, and those without H1 were like the core particle, then a difference in linking number for closed circular DNA should occur depending on whether H1 is present or absent (-2 for the former case and -1 for the latter). To investigate this, chromatin was assembled in vitro from relaxed closed circular DNA (SV40) and core histones at histone to DNA ratios of 0.2 to 0.3 (g/g) and incubated with topoisomerase I to relax supercoils in DNA regions not constrained by protein. Addition of histones H1 + H5 to the chromatin at an ionic strength of 0.1 M, in the presence of polyglutamic acid and topoisomerase I, increased the magnitude of the DNA linking number change, relative to protein-free DNA. No change in the linking number distribution occurred for relaxed protein-free DNA under these conditions. Control experiments indicated that the increase in the absolute value of the DNA linking number change in the chromatin could not be attributed to an increase in the number of nucleosomes per DNA molecule. These data lead to an apparent solution to the linking number problem associated with models of chromatin structure; particles without H1 have linking number change of -1, while those with H1 have -2. These data suggest an even more fundamental role for the chromatosome as the basic repeating unit in most chromatins than previously surmised.

The last year has seen increasing evidence that the structure of DNA itself may not be as monotonous as envisioned from a static view of a Watson-Crick double helical B-form molecule. We reported an alternating phosphodiester backbone conformation for poly(dA-dT) and similar ^{31}P NMR data were obtained by Pael et al. for poly(dG-dC) oligomers at NaCl concentrations over 2.5 M. Rich and colleagues recently determined the crystal structure of a trimer of dG-dC to be a right-handed double helix with alternating phosphodiester geometry, a structure they called Z-DNA.

These workers suggested that Z-DNA might have major functions in vivo and that basic proteins might replace the high NaCl concentrations necessary in vitro to enforce this altered conformation upon DNA. To examine the proposal we have studied the conformation of 145 base pair poly(dG-dC)·poly(dG-dC) in three conditions; i) associated with histone octamers, ii) alone at ionic strength 0.1, and iii) in solutions of over 2.5 M NaCl. The circular dichroism spectrum for the polymer bound to histones differs from that for the free polymer; the difference spectrum is similar to those for native and poly(dA-dT) poly(dA-dT) core particles. Under the first two conditions, the ^{31}P NMR spectrum is symmetric with line widths of 91 and 41 Hz, respectively, at 109.3 MHz. In high salt, two ^{31}P peaks of equal intensity are observed confirming recent

results of Patel et al. and indicating an alternating geometry for the phosphodiester backbond. Using this highly homogeneous DNA, we confirm that the Pohl-Jovin transition is an intramolecular rearrangement, not requiring complete strand separation. The absence of peak splitting in the G-C core particle (limit of detection less than 5%) clearly demonstrates that Z-DNA is not present when this polydeoxyribonucleotide is associated with the inner histones, casting doubt on the chances of its occurring at all in vivo in association with basic proteins.

Significance to Biomedical Research and the Program of the Institute: Elucidation of the structure of the eukaryotic nucleoprotein complex, chromatin, is necessary for understanding the mechanism of gene regulation in higher organisms. Our current studies aim at complete understanding of the structural relationships between histones and DNA to create the nucleosome, the basic structural element of chromatin, and higher orders of chromatin structure.

Proposed Course of Project: Investigations of histone-DNA interactions and the mechanisms of folding of DNA by interaction with the octamer and H1 will continue. Particular emphasis will be directed towards the attempts to recreate with fidelity in vitro the structure of chromatin observed in vivo; this is a mandatory prerequisite to experiments using cloned gene segments to attempt to reconstruct a functional gene as chromatin in vitro from isolated components. We will pursue structural studies of chromatin at different stages of early embryogenesis in sea urchin as a model system for the marked remodeling and functional changes that occur in the genome during the activation of the zygote for development and the earliest stages of determination and differentiation.

Publications:

Bina, M., Sturtevant, J.M., and Stein, A.: Stability of DNA in nucleosomes. Proc. Natl. Acad. Sci. USA, in press.

Shindo, H., Simpson, R.T., and Cohen, J.S.: An alternating conformation characterizes the phosphodiester backbone of poly(dA-dT) in solution. J. Biol. Chem. 254: 8125-8128, 1979.

Simpson, R.T.: Dissection of chromatin structure with nucleases. In Chirikjian, J.G. and Papas, T.S. (Eds.): Gene Amplification and Analysis, V. II. Analysis of Nucleic Acids by Enzymatic Methods. New York, Amsterdam, Elsevier-North Holland, in press.

Simpson, R.T. and Shindo, H.: Conformation of DNA in chromatin core particles containing poly(dA-dT)·poly(dA-dT) studied by ^{31}P NMR spectroscopy. Nucleic Acids Res. 7: 481-492, 1979.

Simpson, R.T. and Shindo, H.: Conformations of 145 base pair length poly(dG-dC)·poly(dG-dC) in solution and in association with histones. Nucleic Acids Res., in press.

Stein, A.: DNA wrapping in nucleosomes. The linking number problem re-examined. J. Biol. Chem., in press.

Stein, A. and Page, D.: Core histone associations in solutions of high salt. An osmotic pressure study. J. Biol. Chem. 255: 3629-3637, 1980.

Stein, A., Whitlock J.P., Jr. and Bina, M.: Acidic polypeptides can assemble both histones and chromatin in vitro at physiological ionic strength. Proc. Natl. Acad. Sci. USA 76: 5000-5004, 1979.

Tack, L.O. and Simpson, R.T.: Location of histone lysyl residues modified by in vitro acetylation of chromatin. Biochemistry 18: 5110-5118, 1979.

Whitlock, J.P., Jr.: The conformation of the chromatin core particle is ionic strength-dependent. J. Biol. Chem. 254: 5684-5689, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15101-09 LNE

PERIOD COVERED
1 October 1979 to 30 September 1980

TITLE OF PROJECT (80 characters or less)

Analysis of the Active and Inactive Portions of the Eukaryotic Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert T. Simpson	Medical Director (R)(T)	LNE NIAMDD
Others:	Grant A. Bitter	Staff Fellow	LNE NIAMDD
	Lawrence W. Bergman	Staff Fellow	LNE NIAMDD

COOPERATING UNITS (if any)

None

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Laboratory of Nutrition and Endocrinology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are developing methodology which will lead to the characterization of components necessary and sufficient for transcription of a specific gene as chromatin, in vitro. Isolation of the 2 µm plasmid of yeast as a minichromosome has been carried out. Conditions which apparently lead to specific initiation on the trp 1 yeast gene in pLC544 have been derived; the presence of cytoplasmic factors and exogenous homologous RNA polymerase II are mandatory for correct transcription of restriction endonuclease truncated templates. Other experiments involve characterization of the structure of chromatin during fertilization and early embryogenesis. The sea urchin sperm core particle bears certain similarities to other core particles, however the presence of variant forms of H2A and H2B leads to marked changes in thermal denaturation of the sperm particle and different accessibilities to cutting by DNAaseI when compared to "typical" core particles from other tissues.

Project Description

Objectives: To investigate the physical and chemical differences between portions of the eukaryotic genome which are transcribed and those which are repressed. To determine the features necessary and sufficient for correct transcription of chromatin and isolated DNA in vitro. To characterize the changes in chromatin structure and function during the transition from inactive gametes to active early embryonic forms.

Methods Employed: HeLa cells and yeast are grown under standard conditions. S. purpuratus, obtained from Paciifc Biomarine, Venice, CA, are maintained at 14° in artificial sea water. Spawning is induced by intracoelomic injection of 0.55 M KCl; eggs and sperm are washed in sea water and stored frozen. Nuclear preparations, digestions with nucleases, and separation of chromatin fragments are done by methods standard in this laboratory except where indicated.

The plasmid pLC544, containing the yeast trp1 gene inserted in pBR313, is grown and amplified in E. coli K-12 under P-2 containment conditions. Plasmid DNA is isolated by CsCl - EtdBr isopycnic centrifugation and restricted with appropriate restriction endonucleases.

Histones are isolated from chicken erythrocyte chromatin or sperm nuclease digests by salt extraction. H1-equivalent histones are fractionated from the inner histones by either 5% perchloric acid extraction or gel filtration in 2.5 M NaCl. Yeast RNA polymerase is prepared as previously described. Cytoplasmic factors used in vitro transcription are fractionated from a high speed supernatant using chromatography on phosphocellulose and DEAE-Sephacel.

DNA and RNA fragments are analyzed by gel electrophoresis under native and denaturing conditions; fragments are localized by staining or autoradiography. Proteins are analyzed by gel electrophoresis using either sodium dodecyl sulfate containing gels or those with Triton X-100, acetic acid and urea.

Physical analyses and analytic digestions with various nucleases are carried out using methods described in previous editions of this project report and those for project Z01 AM 15100-10 LNE.

Major Findings: Long term goals of this research project are to establish conditions for correct transcription of an isolated, defined gene sequence, establish composition and functional features of the same gene isolated as chromatin, and finally, reassemble the chromatin, testing individual components as to their roles in transcriptional regulation. Two of the areas of research covered in the current report are directed at the first two of these goals; we will present results as we now have them, realizing that they are preliminary and may change due to the complexity of the project.

We are developing methology for isolation of plasmid DNA from yeast in the form of a histone-bound minichromosome. Initially, we use the 2 μm plasmid which is normally present in many yeast strains at a moderately high copy

number (about 100 per cell). The plasmid is released by spheroplast preparation using zymolyase and lysis of the spheroplasts. Restriction maps of the plasmid DNA are consistent with those of others, although they are complicated due to the presence of two forms of the plasmid, differing in orientation of the genetic sequence.

This DNA, when sedimented without deproteinization on sucrose gradients, sediments significantly faster than would be expected for protein-free DNA, either circular relaxed or superhelical, suggesting that it is in the form of a minichromosome. Data of others, using blot-hybridization techniques, show that the micrococcal nuclease repeat length for the plasmid sequences is about 200 base pairs, consistent with this interpretation.

The actual plasmid we hope to use for later studies is currently being used only for establishment of the in vitro transcription system. pLC544 contains the trp1 gene sequence inserted in pBR 313. It contains a putative replication origin and replicates in yeast, as well as in host E. coli. Using tryptophan auxotrophs, the presence of the plasmid can be assured. The sequence of the trp1 insert is known from studies in Dr. J. Carbon's laboratory.

In vitro transcription studies using homologous yeast RNA polymerase II, restriction endonuclease truncated templates from plasmid DNA, cytoplasmic factors, and α-^{32}P-UTP have been in progress for several months. In the presence of an S100, material which has mobility on gel electrophoresis about that expected for selective initiation (knowing the position of template cutting and the putative start site for translation) is synthesized by polymerase II. The synthesis is completely abolished by α-amanitin. When factors are partially purified by salt-step chromatography on phosphocellulose and DEAE-Sephacel, synthesis becomes totally dependent on the simultaneous presence of both factors and exogenous RNA polymerase II. Two different truncated templates give products whose relative size is that expected from the known positions of cutting of the two restriction endonucleases. Further characterization of this in vitro transcription system is proceeding currently.

In a different area of research, also just beginning in our laboratory, we are also attempting to evaluate the features that lead to differential gene expression, in this case, in a system undergoing rapid developmental changes. At the time of fertilization in sea urchin, transcriptionally dormant male and female gametes fuse. Within 10 min. the compact sperm nucleus has undergone a 100 fold volume expansion. During the next 10 min, the male pronucleus migrates centrad and fuses with the female pronucleus; at about the time of fusion both nuclei begin RNA synthesis and begin DNA replication in preparation for the first zygotic mitosis. It is known that sea urchin sperm and egg histones differ from those of the adult; further, several different sets of H1, H2A, and H2B histones associate with chromatin during differentiation from zygote to gastrula. Essentially nothing is known of the structure of chromatin in early development. I have decided to make delineation of structure-function relationships in fertilization and early embryogenesis a major area of research for the next several years.

In beginning our studies in this area, we have characterized the structure of the chromatin core particle of sea urchin sperm. Sea urchin sperm contains forms of H1, H2A and H2B which differ strikingly from those of adult tissues. The core particle of S. purpuratus sperm contains 145 base pairs of DNA and equal amounts of the four smaller histones. It sediments at 11 S and has a circular dichroism spectrum similar to that of particles containing more typical histones. The sperm core particle undergoes a shape change at low ionic strength, as observed for calf thymus and chicken erythrocyte core particles.

In contrast to these similarities, the melting profile of the sperm core particle is quite different from that of erythrocyte; both the reversible transition and the irreversible denaturation of the core particle occur at higher temperatures (68-73° and 78.5°, respectively). The sperm core particle is digested by DNAase I more slowly than other core particles. DNAase I cutting site maps for the sperm core particle reveal the same basic organization of DNA by these variant histones; however, features of the map differ significantly from the map for other core particles. Particularly, the sites at 20 and 40 bases from the 5' end of the DNA, highly accessible in other particles, are very infrequently cut in the sperm particles. These data demonstrate modulation of the canonical core particle structure by the variant H2A and/or H2B present in sea urchin sperm chromatin.

Derived from data we have obtained in the last several years, we have developed an operational model for the mechanism whereby transcription of DNA might proceed through a nucleosome, where the nucleic acid is tightly wrapped around an octamer of histones. The model contains three essential elements, all of which have been justified by several types of study in our laboratory: (1) transcription begins by DNA unwinding from the end of the core particle segment for about 20-25 base pairs, (2) a change in DNA (or DNA-RNA hybrid) geometry to A-form further destablizes the interaction of DNA with the histones, and (3) stability of histone octamer-DNA interactions becomes so low that the octamer is transferred to a new binding position, either to another nucleosome in the vicinity of the transcription complex or to an acidic chromosomal protein. Following passage of polymerase, the system relaxes to the lowest energy, unperturbed state with all DNA associated with histones as nucleosomes. In the Bridgeman sense of operationalism, we do not view the model as a representation of fact, but do feel it synthesizes a number of experimental observations into a unit; further it suggests experiments which will confirm, refine, or refute the mechanistic proposal.

Significance to Biomedical Research and the Program of the Institute: One of the most direct approaches to understanding the basis for transcriptional regulation is comparison of the composition and structure of transcribed with repressed segments of chromatin. This separation has proven to be difficult if not impossible with current tools and unique genes. We therefore, have concentrated on probing the structure of active chromatin regions with a series of small molecules, hormones, nucleases, and polymerases. Progress in these areas will aid in achievement of our eventual goal, description of the mechanism of transcriptional regulation in animal cells and thereby the features responsible for the stable differentiated state of a normal mature cell. Under-

standing of these features will facilitate understanding of neoplastic transformation and normal developmental and differentiation.

Proposed Course of Project: We will develop a correct in vitro transcription system using the pLC544 plasmid containing the yeast trp1 gene. We will isolate and characterize the 2 μm plasmid of yeast. Using the expertise acquired with these systems, we will characterize the structure and function of a circularized trp1 fragment propagated in yeast. Studies of the state of chromatin during early embryogenesis in sea urchin will continue with the goal of relating composition, structure, and function during major alterations in genetic expression in early embryogenesis.

Publications

Simpson, R.T.: Mechanism of a reversible, thermally induced conformational change in core particles. J. Biol. Chem. 254: 10123-10127, 1979.

Simpson, R.T. and Bergman, L.W.: Structure of sea urchin sperm chromatin core particle. J. Biol. Chem., in press.

Simpson, R.T. and Stein, A.: Random protection of single cut restriction endonuclease sites in SV40 mini-chromosomes assembled in vitro. FEBS Letters 111: 337-339, 1980.

Simpson, R.T., Stein, A., Bitter, G.A. and Kunzler, P.: Chromatin structure and function: a model for transcription of nucleosome-associated DNA. In Smulson, M. and Sugimura, T. (Eds.): Proceedings of the Fifth International Conference on Novel ADP-ribosylations of Regulatory Enzymes and Proteins. New York, Amsterdam, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 15102-20 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Study of Ribonuclear (Barnase) and its Inhibitor (Barstar) from Bacillus amyloliquefaciens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Robert W. Hartley Research Physicist LNE NIAMDD

COOPERATING UNITS (if any)
D.M. Blow, Dept. of Biophys., Imperial College, London; Chothia, Dept. Chem., University College, London; G.C. Dodson, Dept. Chem., Univ. of York; and R.S. Sheppard, Lab. of Molecular Biology, MRC, Cambridge

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

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Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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0.4	0.2	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two proteins, barnase, the extracellular ribonuclease of Bacillus amyloliquefaciens, and barstar, its intracellular inhibitor, are being developed as a model system for the study of protein folding and protein-protein interactions. Amino acid sequence determination and X-ray structure characterization are being carried out along with thermodynamic studies of folding reactions. A peptide complementation system for barnase has been developed which will be used to investigate the roles of specific residues in both folding and enzymatic activity.

Project Description

Objectives: To further characterize and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques. To develop methods of culture, genetic manipulation and genetic analysis so that the organism and its proudct enzyme-inhibitor pair may be applied to a broad study of structure, function and synthesis.

Specific objectives are: 1) the determination of the amino acid sequence and three-dimensional structure (by X-ray diffraction) of both proteins and te structure of their complex; 2) thermodynamic and kinetic analysis of structural transitions produced in both proteins by heat or denaturating reagents 3) analysis of the folding and activity of both by means of peptide complementation systems analogous to those being used for the study of pancreatic ribonuclease and micrococcal nuclease; 4) all of the above with chemically and/or genetically modified sequences.

Methods Employed: B. amyloliquefaciens (strain H and derivatives and several others) are grown in synthetic media. Barnase is absorbed from the culture medium onto phosphocellulose, barstar is extracted from the cells. Their purifications make use of ion exchange, gel-permeation and affinity chromatography.

Work on the determination of amino-acid sequences involves amino acid analysis, enzymatic digestions, peptide separations and peptide sequencing by dansyl-Edman and difference-Edman techniques. Peptide separation and amino acid analysis are by automated ion-exchange chromatography using a modified JEOL analyzer equipped for detection with Roth's orthophthaldehyde reagent. Dansyl amino acids are identified by thin-layer chromatography on polyamide layers.

The reversible transitions undergone by barnase and barstar and their derivatives under the influence of temperature changes or denaturing agents are studied primarily by ultraviolet and fluorescence spectroscopy, but also by optical rotatory dispersion, circular dichroism, exclusion chromatography with the use of proteolytic enzymes and by equilibrium binding studies. These techniques are also used to study complementation reactions between natural and synthetic peptide sequences of the two proteins as well as conformational properties of each peptide alone. Studies of the peptide derivatives also include use of antibodies, chromatography, crystal growth, etc.

Crystals are grown in hanging drops (10 µl) suspended over suitable solvents. Isomorphous crystals containing heavy atoms are prepared by soaking crystals in solutions of appropriate heavy atom compounds. The three dimensional structure of barnase is being determined by methods of X-ray crystallography.

The Merrifield procedure has been used to synthesize portions of the barnase sequence.

Major Findings: X-ray diffraction work has yielded a map of barnase. It is not totally interpretable, though in regions it looks very good and some specific residues can be identified. In obtaining this map an iodinated derivative was used which was iodinated after crystal growth. It was interesting to find that the three molecules of the crystalline assymetric unit were not all derivatised in the same way, explaining the ambiguity seen in attempts to identify the modified tyrosine chemically.

Significance to Biomedical Research and the Program of the Institute: The small size, stability, easily measured enzyme activity and absence of disulfide bridges in barnase make it an excellent choice for a long-range study of the relation between the sequence of an enzyme and its structure and function. The inhibitor is an even simpler protein and should be useful in a similar fashion. Together, they offer a model system for the study of protein-protein interaction.

The nature and extent of the thermal transition of barnase gives strong support to the concept that the three-dimensional structure of a protein may be completely determined by its amino acid sequence. The simplicity and all-or-none nature of the transition should make it useful for thermodynamic analysis of the factors involved in maintaining a folded protein structure. The production of both of these proteins by the same organism is also of biological interest.

The discovery of homology between prokaryotic and eukaryotic ribonucleases greatly extends the significance of this work. Study of the whole group of enzymes should help our understanding of evolutionary relationships on the broadest scale. Comparisons of their sequences and structures may contribute substantially to the solution of the sequence-directed-folding problem.

Proposed Course of the Project: Collaboration with the authors in England on the X-ray structure and on peptide complementation will continue.

Publications

Hartley, R.W.: Homology between prokaryotic and eukaryotic ribonucleases. J. Molecular Evolution, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15104-08 LNE

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on Eukaryotic DNAs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: George Rushizky Research Chemist LNE NIAMDD

COOPERATING UNITS (if any)

Pilot Plant, LNE, NIAMDD
Dr. F. deFillipes, NIAID

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

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1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project involves the fractionation and isolation of large DNA fragments of biological activity, such as the origin of replication of vaccinia virus.

PHS-6040

Project Description

Objectives: To isolate specific fragments from large DNAs, labeled or in amounts sufficient for studies of their structure and biological function. Emphasis is placed on improvement of existing methods or development of new ones.

Methods Employed: DNAs were prepared from phages and vaccinia virus grown by standard procedures in bacteria and HeLa cells, respectively. To determine the site of the origin of DNA replication involves pulse-labeling of infected cells, fractionation of lysed extracts, removal of cellular DNA, isolation of 70 S DNA by sucrose gradient centrifugation, restriction endonuclease hydrolysis with various enzymes and fractionation of fragments so obtained, and examination of labeling patterns.

Major Findings: While the virus is believed to by synthesized exclusively in the cytoplasm, more full-length V-DNA was found in the nuclear- than in the cytoplasmic fraction. To rule out contamination of the nuclear fraction by material leached from the cytoplasm, various approaches were used. None of these could rule out the presence of V-DNA in the nuclear fraction due to DNA bound to nuclear membranes on the cytoplasm side. However, by labeling patterns of restriction fragments, the cytoplasm contained uniformly labeled viral DNA, while in the nuclear fraction labeling was not uniform.

Work is in progress to confirm the above results. To this end, V-DNA hydrolysis by other restriction enzymes was examined. Bgl I cuts V-DNA into four fragments which are readily separated by sucrose gradient centrifugation. The order of these fragments along the DNA molecule was determined. Sac I yields 7 fragments from V-DNA more alike in size (and more readily determined) than those from Hind III digests. This project is not completed.

With the departure of Krishna Baksi (Annual Report 78-79), studies on the fractionation of DNA restriction fragments on base-specific adsorbents have ended.

Significance to Biomedical Research and the Program of the Institute: Involvement of the cellular nucleus in viral DNA synthesis implies a unique transcription or processing step or a messenger function that is transient in that 6 h after infection very little V-DNA remains in the nuclear fraction, with the bulk of the V-DNA located and having been replicated in the cytoplasm. Clearly, assignment of the function of nuclear V-DNA also bears on the method of replication of it.

Proposed Course of the Project: Goals include the determination of the site of origin of DNA replication, and a characterization of the role of nuclear V-DNA.

Publications

Baksi, K. and Rushizky, G.W.: Purification of the restriction endonuclease Pal I. Analytical Biochemistry 99: 207-212, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15105-04 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

A Developmental Transition in Mosses as a Model for the Study of Eukarytoic Genome Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Robert W. Hartley Research Chemist LNE NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	0.8	0.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The developmental transition of the moss gametophyte from filamentous protonema to leafy gametophore is being developed as a model for eukaryotic systems in which large blocks of genes are switched on or off. The transition (in Nanomitrium austinii) may be induced by cytokinins or, under certain conditions and reversibly, by changes in temperature.

The coordinate control of the two structural genes of ribulose bis-phosphate carboxylase is also being investigated in these organisms.

PHS-6040

Project Description

Objectives: In their development, mosses pass through two distinct generations, represented by the haploid gametophyte and the diploid sporophyte. The haploid generation is further divided into at least two distinct morphological stages, the mature "leafy" gametophore being preceded by the purely filamentous protonema. The transition between these two forms may, in some cases, be controlled by temperature or by plant growth substances of the cytokinin (6-N substituted aminopurine) type. The current objective is to bring into aseptic culture one or several moss species and study this discrete transition as an instance of coordinated gene-switching in a typical eukaryote. The system will also be used to study the enzyme ribulose bis-phosphate (RuBP) carboxylase. This enzyme (by a large factor the most abundant protein on earth) is of great practical importance to agriculture, but interest in the present context is in its mode of synthesis. Its two different subunits are coded in different genomes and synthesized in different cellular compartments, presenting a pretty problem in coordinated control of genetic information.

Methods Employed: Aseptic cultures are obtained by spreading the contents of surface-sterilized spore capsules onto suitable agar media. Culture thereafter is largely by standard microbiological techniques.

Protonemal tissue is obtained by growing it on a seimpermeable membrane (overlying agar) from which it can be scraped, or by deep liquid culture in volumes up to 10 L. Large numbers of individuals are obtained by chopping up protonemal tissue in a Waring blender. Protonemal fragments are mutagenized with MNNG or UV light and a variety of mutant searching techniques carried out. A system has been devised for testing large numbers of mutagenized cells for auxotrophy. It is analogous to replica plating but involves photographic recording and the transfer of colonies supported by a membrane from plate to plate. Protonema are also grown continuously in suspension in a chemostat especially devised for handling filamentous organisms.

Protoplasts (osmotically fragile cells lacking any cell well) are obtained by treatment with a commercial cellulase preparation (Driselase) obtained from the basidiomycete Irpex lacteus.

Proteins are extracted from protonema or from lysed protoplasts and separated and studied by various chromatographic and/or electrophoretic techniques.

The RuBP-carboxylase is assayed by a method involving the fixation of C-14 labelled CO_2.

Significance to Biomedical Research and the Program of the Institute: The moss protonema-gametophore transition offers a convenient system for approaching the general problem of gene control in eukaryotes, particularly in the developmental aspect where large sections of the genome are turned on or off.

The RuBP carboxylase system offers access to another gene-control system in a typical eukaryote amenable to microbiological techniques.

Proposed Course of Project: Development of genetic background information with which to study developmental mutations and the search for such mutations will continue.

Identification and isolation of proteins unique to the particular developmental states will be attempted.

The biochemical fate of the effective cytokinins will be investigated, including a search for specific receptors.

The RuBP carboxylase will be further characterized and methods for obtaining mutations in the genes involved in its synthesis investigated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 15200-20 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on Folic Acid (Dihydrofolate Reductase)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Dr. Bernard T. Kaufman Research Chemist LNE NIAMDD

COOPERATING UNITS (if any)

Dr. James H. Freisheim, Department of Biological Chemistry, University of Cincinnati, College of Medicine, Cincinnati, Ohio

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Vitamin Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous studies from this laboratory have led to the development of affinity chromatographic procedures for the purification of relatively large amounts of homogeneous, cosubstrate-free, crystalline dihydrofolate reductase from chicken liver. Thus sufficient enzyme protein from liver, the main site of dihydrofolate reductase activity in animals, was now available for sequencing. The S-carboxymethylated protein was subjected to cleavage by cyanogen bromide which produced five fragments. Sequences and ordering of the cyanogen bromide fragments were established by means of automated sequencer analysis of the fragments and from smaller peptides generated by proteolysis with trypsin and staphyloccal protease. The complete amino acid sequence comprising the covalent structure of the single polypeptide chain consists of 189 residues of molecular weight 21651.

PHS-6040

Project Description

Objectives: The interaction of drugs, substrates and cofactors with dihydrofolate reductase continues to be of considerable pharmacological and biochemical interest since this enzyme appears to be the site of activity of the powerful and useful chemotherapeutic agent, methotrexate. Dihydrofolate reductase which is concerned with the conversion of the essential vitamin, folic acid, to the biologically active cofactor, tetrahydrofolate, has a central role in cellular proliferation. For this reason we have continued to study the isolation, purification and properties of the reductases from various sources, particularly animal livers. Although the enzymes from two vertebrate livers (beef and chicken) have been purified to apparent homogeneity, many intriguing problems concerning the nature of the purification process remain to be examined. These include the nature of the unknown substances which bind to the affinity columns, the multiple peaks of activity observed on isoelectric focusing and the variation in isoelectric points of the reductases from various animal and bacterial species. Using the highly purified reductases from bovine and chicken liver, investigations are continuing on the structure (amino acid sequence and X-ray crystallography) and function of these enzymes.

Methods Employed: Affinity chromatography, using a chromatographic system based on the drug methotrexate coupled to sepharose, has been used to absorb dihydrofolate reductase from relatively crude liver extracts. However in order to obtain the homogeneous, substrate-free reductase, additional purification involving gel filtration and isoelectric focusing are required. Structural studies on the enzyme involved the use of automatic amino acid analysis using the general procedures of Moore and Stein. Sequence analyses involving Edman degradation were performed with an automated sequenator. The phenylthiohydantoin-amino acid derivatives sequentially liberated were identified by gas chromatography after trimethylsilylation. Additional methods of chemical and enzymatic analysis involved the use of spectrophotometry and fluorescence spectroscopy.

Major Findings: Previous studies from this laboratory have led to the development of affinity chromatographic procedures for the purification of relatively large amounts of homogeneous, cosubstrate-free, crystalline dihydrofolate reductase from chicken liver. Thus sufficient enzyme protein from liver, the chief site of dihydrofolate reductase activity in animals, was now available for sequencing. The S-carboxymethylated protein was subjected to cleavage by cyanogen bromide which produced five fragments. Sequences and ordering of the cyanogen bromide fragments were established by means of automated sequencer analysis of the fragments from smaller peptides generated by proteolysis with trypsin and staphyloccal protease. The complete amino acid sequence comprising the covalent structure of the single polypeptide chain consists of 189 residues of molecular weight 21651 as is shown below.

Since the completion of this structure, the sequence of three other animal reductases have been reported, bovine liver and two tumor cells, L1210 and sarcoma 180 in addition to the bacterial reductases previously reported, E. coli, S. facium, and L. casei. However only the L. casei and chicken liver

have been crystallized with the chicken liver enzyme the only one crystallized free of methotrexate. The X-ray structural studies on the chicken liver dihydrofolate reductase are progressing well. Current studies are also being directed toward attempting to relate the unusual properties of these enzymes particularly those from chicken and beef liver to the now known structure of the enzyme.

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  1  VAL-ARG-SER-LEU-ASN-SER-ILE-VAL-ALA-VAL-CYS-GLN-ASN-MET-GLY-ILE-GLY-LYS-ASP-GLY-ASN-LEU-PRO-TRP-PRO-
 26  PRO-LEU-ARG-ASN-GLU-TYR-LYS-TYR-PHE-GLN-ARG-MET-THR-SER-THR-SER-HIS-VAL-GLU-GLY-LYS-GLN-ASN-ALA-VAL-
 51  ILE-MET-GLY-LYS-LYS-THR-TRP-PHE-SER-ILE-PRO-GLU-LYS-ASN-ARG-PRO-LEU-LYS-ASP-ARG-ILE-ASN-ILE-VAL-LEU-
 76  SER-ARG-GLU-LEU-LYS-GLU-ALA-PRO-LYS-GLY-ALA-HIS-TYR-LEU-SER-LYS-SER-LEU-ASP-ASP-ALA-LEU-ALA-LEU-LEU-
101  ASP-SER-PRO-GLU-LEU-LYS-SER-LYS-VAL-ASP-MET-VAL-TRP-ILE-VAL-GLY-GLY-THR-ALA-VAL-TYR-LYS-ALA-ALA-MET-
126  GLU-LYS-PRO-ILE-ASN-HIS-ARG-LEU-PHE-VAL-THR-ARG-ILE-LEU-HIS-GLU-PHE-GLU-SER-ASP-THR-PHE-PHE-PRO-GLU-
151  ILE-ASP-TYR-LYS-ASP-PHE-LYS-LEU-LEU-THR-GLU-TYR-PRO-GLY-VAL-PRO-ALA-ASP-ILE-GLN-GLU-GLU-ASP-GLY-ILE-
176  GLN-TYR-LYS-PHE-GLU-VAL-TYR-GLN-LYS-SER-VAL-LEU-ALA-GLN
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Significance to Biomedical Research and the Program of the Institute: Dihydrofolate reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of DNA via the biosynthesis of thymidylate. Thus an inhibition of dihydrofolate reductase causes a depletion of tetrahydrofolate leading in turn to a deficiency in thymidylate. Such a deficiency can lead to a cessation of growth in rapidly proliferating cells such as tumor cells. Since a variety of dihydrofolate reductase inhibitors are clinically useful as anti-bacterials, anti-protozoals, immunosuppressants and anti-neoplastic agents, an understanding of the structure and function of this enzyme would be of basic importance in both normal metabolism and clinical medicine. As yet, many of these basic questions remain to be answered.

Proposed Course of Project: Now that the basic physical properties of the homogenous proteins are known to both the beef and chicken dihydrofolate reductases, studies are continuing towards the elucidation of the structure of the enzymes in terms of the amino acid sequences and three dimensional structure via X-ray analysis. In addition, studies will continue to be directed toward the investigation of the interaction of drugs and cofactors with the purified reductases.

Publications

Barbehenn, K.E. and Kaufman, B.T.: Alteration of the properties of chicken liver dihydrofolate reductase as a result of modification by tetrathionate. J. Biol. Chem. 255: 1978-1984, 1980.

Blankenship, D.T., Kaufman, B.T. and Freisheim, J.H.: Primary structure of chicken liver dihydrofolate reductase. Biochemistry 19: 667-678, 1980.

Kaufman, B.T., Kumar, A.A. and Freisheim, J.H.: Activation of bovine and chicken liver dihydrofolate reductase and its relationship to a

specific cysteine residue in their N-terminal amino acid sequences. J. Biol. Chem. (in press, July).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15201-01 LNE

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Ligand Interactions with Dihydrofolate Reductase-Physico-Chemical Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Dr. S. Subramanian	Visiting Scientist	LNE	NIAMDD
Other:	Dr. B.T. Kaufman	Research Chemist	LNE	NIAMDD

COOPERATING UNITS (if any)
Dr. H. Shindo, LCP, NIAMDD, NIH

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Vitamin Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cibacron blue F3GA binds to dihydrofolate reductase from chicken liver and Lactobacillus casei in an 'electrostatic mode' and 'hydrophobic mode' respectively. Although the enzyme has a 'pseudo-dinucleotide fold', the blue dye does not bind at the proper nucleotide binding site to the enzyme from either species.

Salt (NaCl) acts as an activator for the reaction catalyzed by dihydrofolate reductase (from chicken liver) if NADPH is used as the coenzyme and as an inhibitor if NADH is used as the coenzyme. ^{35}Cl NMR studies reveal that this dichotomy in the kinetics is a result of the mutual exclusivity of chloride and NADPH binding at the active site of the enzyme while chloride and NADH binding to the enzyme are mutually inclusive.

Project Description

Objectives: (1) To investigate the binding of cibacron blue F3GA to dihydrofolate reductase from bacterial and mammalian sources to understand the differences and similarities in the properties of the enzyme from different species.

(2) To study the interaction of chloride ion with the enzyme by ^{35}cl NMR in order to understand the kinetic behavior of the enzyme in presence of NaCl.

Methods Employed: FT NMR Spectroscopy, Ultraviolet and visible spectroscopy.

Major Findings: (1) Cibacron blue F3GA, a dye probe, used as an affinity column in the immobilized form binds to dihydrofolate reductase from chicken liver in the dihydrofolate site and the interactions of the dye with the amino acid side chains are predominantly ionic. In contrast, the blue dye binds to the enzyme from Lactobacillus casei in a totally hydrophobic fashion in a site which overlaps the nicotinamide mononucleotide moiety of NADPH and the dihydrofolate binding site.

(2) Chloride bound to dihydrofolate reductase can be displaced by binding NADPH to the enzyme stoichiometrically. However, folate, dihydrofolate or methotrexate binding do not seem to affect chloride binding. NADH, by itself, does not affect chloride binding but in conjunction with methotrexate it displaces chloride from the enzyme.

Significance to Biomedical Research and the Program of the Institute: Dihydrofolate reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of DNA via the biosynthesis of thymidylate. Thus an inhibition of dihydrofolate reductase causes a depletion of tetrahydrofolate leading in turn to a deficiency in thymidylate. Such a deficiency can lead to a cessation of growth in rapidly proliferating cells such as tumor cells. Since a variety of dihydrofolate reductase inhibitors are clinically useful as anti-bacterials, anti-protozoals, immunosuppressants and anti-neoplastic agents, an understanding of the structure and function of this enzyme would be of basic importance in both normal metabolism and clinical medicine. As yet, many of these basic questions remain to be answered.

Proposed Course of Project: Now that the basic physical properties of the homogenous proteins are known for both the beef and chicken dihydrofolate reductases, studies are continuing towards the elucidation of the structure of the enzymes in terms of the amino acid sequences and three dimensional structure via x-ray analsysis. In addition, studies will continue to be directed toward the investigation of the interaction of drugs and cofactors with the purified reductases.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER New No. Z01 AM 15202-01 LNE formerly Z01 AM 33055-05 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Thermodynamics of Dehydrogenase-Ligand Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: S. Subramanian Visiting Scientist LNE NIAMDD

COOPERATING UNITS (if any)
Dr. H.F. Fisher, Veterans Administration Hospital,
Kansas City, MO 64128

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Vitamin Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The reaction catalyzed by bovine L-glutamate dehydrogenase is subject to allosteric activation by ADP. We have measured the thermodynamic parameters (ΔG^o, ΔH^o, ΔS^o, and ΔC_p^o) of the formation of the various possible binary and ternary complexes formed between the enzyme, NADPH, and either ADP or its analogs, adenosine, AMP, and ATP. ΔH^o and ΔC_p^o have been measured by flow calorimetry; ΔG^o values obtained by calorimetry itself, difference spectroscopy, or gel filtration have been selected on the basis of accuracy under the conditions required for the formation of each complex. The data are interpreted in terms of "interaction parameters," the differences between the thermodynamic parameters of the formation of a ternary complex and the sum of those of the two related binary complexes. Both adenosine and ATP appear to loosen the binding of NADPH by simply preventing a subsite interaction of NADPH. AMP appears to have only minor and probably secondary effects. The negative effect of the binding of ADP on that of NADPH, however, involves the formation of a new interaction, which is exothermic, entropy compensated, has a moderately large negative ΔC_p, and does not occur in either binary complex.

Project Description

Objectives: (1) To obtain thermodynamic parameters for the binding of NADPH and ADP or its analogs with bovine liver glutamate dehydrogenase forming binary and ternary complexes and analyze the data in the light of activation of the catalytic reaction by ADP and its analogs (2) To investigate the nature of interactions in the complex formation between horse liver alcohol dehydrogenase and NADH or NAD with several substrate analogs.

Methods Employed: Batch and Flow Microcalorimetry, Ultraviolet Spectroscopy.

Major Findings: (1) The thermodynamic parameters for the formation of various possible binary and ternary complexes between the enzyme, NADPH and either ADP or its analogs adenosine, AMP and ATP have been obtained. The differences in the parameters for the formation of a ternary complex and the sum of those of the two related binary complexes are termed "interaction parameters" and these are the meaningful parameters used to describe the ligand-enzyme interactions. Both adenosine and ATP loosen the binding of NADPH by interfering with the interaction of an NADPH moiety with an enzyme subsite. AMP has only minor effect. The antagonistic effect of ADP on NADPH binding, however, involves a new interaction in the ternary complex which does not occur in either binary complex and is exothermic and entropy compensated.

(2) When the ternary complex of liver alcohol dehydrogenase-NADH-isobutyramide is formed the fluorescence of Trp-314 is quenched and it was proposed by other workers that Try-286 gets ionized upon complex formation which is responsible for the fluorescence quenching. UV spectral features were pointed out as evidence for Tyr-286 ionization. We studied the spectra of several complexes carefully and the spectra of coenzymes in several solvents and conclude that the spectral features thought to indicate Tyr-286 ionization actually arise from the coenzymes bound in an ionic environment in liver alcohol dehydrogenase and that the tyrosine 286 does not appear to ionize in concordance with our previous thermodynamic study on the enzyme (Cf. Publication #1 next page).

Significance to Biomedical Research and the Program of the Institute: This project is designed to obtain a better understanding of the structure-function-interaction thermodynamics relationships in dehydrogenases. The basic knowledge gained thereby is helpful in unraveling the mechanistic aspects of glycolytic and metabolic enzymes.

Publications:

Subramanian, S. and Ross, P.D.: The enthalpy of protolysis of liver alcohol dehydrogenase upon binding nicotinamide adenine dinucleotide. J. Biol. Chem. 254, 7827-7830 (1979).

Fisher, H.F., Subramanian, S., Stickel, D.C. and Colen, A.H. The thermodynamics of a negatively interacting allosteric effector system: The glutamate dehydrogenase NADPH-ADP complexes. J. Biol. Chem. 255, 2509-2513 (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15300-15 LNE

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Studies Related to Nutrition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. G. Bieri, Chief, Nutritional Biochemistry Sec., LNE, NIAMDD

Other: T. J. Tolliver, Chemist, Nutritional Biochemistry Sec., LNE, NIAMDD
M.C. McKenna, Staff Fellow, Nutritional Biochemistry Sec., LNE, NIAMDD

COOPERATING UNITS (if any)
Laboratory of Vision Research, NEI
Clinical Oncology Program, Surgery Branch, NCI

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Nutritional Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies with rats and chicks have shown that low dietary levels of retinoic acid (vitamin A acid) markedly reduce the plasma and tissue concentrations of α-tocopherol (vitamin E) compared to animals receiving the same dietary amount of retinol (vitamin A). This effect of retinoic acid appears to be due primarily to an impairment in intestinal absorption, as shown by determining the α-tocopherol in lymph after radioactive α-tocopherol was given into the duodenum. There was no effect of dietary retinoic acid on plasma triglycerides, cholesterol or total lipids. In other studies, the utilization of vitamins A and E when given in total parenteral nutrition solutions was determined in rats. It was intitially found that the plastic tubing usually used clinically for this technique completely adsorbed the solubilized vitamins A and E. Only by greatly increasing the concentration of these vitamins so that saturation of the tubing occurs has it been possible to deliver the vitamins intravenously to the animals.

PHS-6040
(Rev. 10-76)

Objectives: To determine the nutritional, biochemical and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study nutritional effects on the tissue levels of various metabolites.

Methods Employed: Rats, hamsters and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical and enzymological changes in the animal, its tissues and excreta.

Major Findings: We observed that rats fed two different forms of vitamin A - retinol and retinoic acid - had markedly different plasma levels of α-tocopherol (vitamin E). Retinol is the normal dietary form of vitamin A, whereas retinoic acid is considered to be a normal metabolite that supports all biochemical functions except those of vision and reproduction. Weanling rats were fed a complete purified diet with either retinol or retinoic acid at 4 mg/kg diet. This is a low, normal amount of vitamin A. After 2-4 weeks the rats were bled and their plasmas analyzed for retinol and α-tocopherol simultaneously by a high pressure liquid chromatography procedure developed in this laboratory. In as short a time as two weeks, feeding retinoic acid resulted in a 50% reduction of plasma α-tocopherol when compared to rats fed retinol. Analyses of liver and adipose tissue showed a similar reduction. This effect of retinoic acid was also demonstrated in chicks. Studies to elaborate the mechanism of this vitamin interaction showed that retinoic acid did not accelerate the loss or destruction of endogenous α-tocopherol. Using ^{3}H-α-tocopherol, it was found in both rats and chicks that the apparent intestinal absorption of α-tocopherol is impaired when retinoic acid is in the diet. In chicks fed retinoic acid, the percent of dose appearing in the blood 2.5 hours after oral administration of labeled α-tocopherol was only 22% that of chicks fed retinol. In rats with a mesenteric lymph cannula, the percent of dose recovered 6 hours after intraduodenal administration of labeled α-tocopherol was reduced by one-third by feeding retinoic acid. Analyses by thin layer chromatography of the recovered radioactivity revealed slightly more oxidative destruction of α-tocopherol in rats fed retinoic acid, but the difference was not enough to account for the magnitude of the difference in plasma α-tocopherol concentration. Analyses revealed that there were no differences in plasma triglycerides, cholesterol or total lipids in rats fed retinol vs. retinoic acid. (Other investigators have shown that higher dietary levels of retinoic acid cause an elevation of plasma triglycerides). Examination of the intestinal mucosa by electron microscopy revealed no morphological differences due to feeding retinoic acid (Dr. W. G. Robison, Jr., NEI). The mechanism by which low intakes of retinoic acid interfere with α-tocopherol absorption is being investigated.

In other studies (Dr. McKenna), the utilization of vitamins A and E in total parenteral nutrition in rats was investigated. A surgical technique for feeding rats totally by vein was developed for determining the maintenance of plasma and tissue levels of these two vitamins (with the advice of Dr. Martin Popp, NCI). It was found initially that these two fat-soluble

vitamins, when dispersed in the aqueous parenteral nutrition solutions customarily used clinically, were almost totally adsorbed by the plastic tubing used for intravenous infusion delivery. Studies using model systems have shown that by elevating the vitamin concentration five to ten fold, saturation of the tubing occurs after 2-4 hours and the vitamins then are delivered at a constant rate to the animal. Experiments are currently underway to determine the tissue retention of vitamins A and E given parenterally for two weeks or longer.

Publications:

(1) Bieri, J. G., Tolliver, T. J., Robison, W. G., Jr., and Kuwabara, T.: Lipofuscin in vitamin E deficiency and the possible role of retinol. Lipids 15(1): 10-13, 1980.

(2) Bieri, J. G.: An overview of the RDAs for vitamins. J. Am. Diet. Asso. 76(2): 134-136, 1980.

(3) Munro, H. N., Bieri, J. G., Briggs, G. M., Butterworth, C. E., Leveille, G. A., Mertz, W., Owen, G. M., Pitkin, R. M., and Sauberlich, H. E.: Recommended Dietary Allowances -- ed. 9. Washington, D.C., National Research Council, National Academy of Sciences, 1980.

(4) Carter-Dawson, L., Tanaka, M., Kuwabara, T., and Bieri, J.G.: Early corneal changes in vitamin A deficient rats. Exp. Eye Res. 30: 261-268, 1980.

(5) Catignani, G. L. and Bieri, J. G.: Ineffectiveness of testosterone and FSH in maintaining the vitamin A deficient rat testis. Nutr. Metab., 1980 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15302-10 LNE

PERIOD COVERED: October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Studies of Hepatic and Intestinal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. H.G. Windmueller	Research Chemist	LNE NIAMDD
OTHER:	Dr. A.-L. Wu	Staff Fellow	LNE NIAMDD
	Mr. A.E. Spaeth	Chemist	LNE NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH: Laboratory of Nutrition and Endocrinology

SECTION: Nutritional Biochemistry Section

INSTITUTE AND LOCATION: NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mucosal epithelial cells of the small intestine have been identified as a major site for the metabolism of circulating glutamine. The glutamine nitrogen is returned to the circulation as ammonia, alanine, proline, and citrulline. The first three products are utilized by the liver and, in fasted rats, provide about 30% of the nitrogen for hepatic urea biosynthesis. The intestinal citrulline is utilized by the kidneys for the biosynthesis of arginine, 80% of which is released back into the circulation as the major source of endogenous arginine for protein synthesis in all tissues. Liver releases neither citrulline nor arginine under physiological conditions. Small intestine has also been identified as a major source of two of the plasma apolipoproteins, apolipoproteins A-I and A-IV. Production of these proteins by intestine is not dependent upon intestinal fat transport. During fat transport, these proteins reach the blood mainly via intestinal lymph in association with chylomicrons. In the absence of fat transport, however, a larger proportion of these proteins is released directly into the blood, by-passing the lymph.

Objectives: To increase our understanding of the biochemical transformations occurring in the liver and small intestine. To define the nutritional requirements of these organs, to study the effects of hormones and nutritional variables, and to study the physiological relationship of these organs to other tissues of the body.

Methods Employed: Surgical and biochemical techniques are used. Surgically prepared animal preparations include isolated, perfused rat livers; isolated vascularly perfused rat small intestine; isolated segments of rat small intestine autoperfused in situ; supra-diaphragmatic rat preparations; and intact animals with various in-dwelling catheters -- in the duodenum, mesenteric lymph duct, bile duct, and jugular vein. The metabolic and transport activity of the organs, isolated or in situ, are studied by a combination of biochemical and radiochemical techniques. The fate of labeled metabolites is quantitatively determined and the concentration of various blood and tissue metabolites is measured enzymatically. Various blood and lymph proteins are isolated by a combination of ultracentrifugal and electrophoretic methods, and their rates of biosynthesis determined from the rates of incorporation of appropriate radioactively-labeled precursors. Also, tissue enzymes are localized, isolated and characterized.

Major Findings: (A) Role of the small intestine in whole body nitrogen metabolism. Earlier work from our laboratory demonstrated that mucosal epithelial cells of the small intestine in most animals, including primates, are a major site for the metabolism of circulating glutamine, the most abundant amino acid in the blood and the main end-product of nitrogen metabolism in skeletal muscle. In the small intestine, oxidation of the glutamine carbon provides an important source of energy; in rats in the postabsorptive state, 35% of the respiratory CO_2 produced by the intestine was shown to be derived from the metabolism of glutamine. We have now determined the metabolic fate of the glutamine nitrogen atoms. Using an autoperfused preparation of rat jejunum developed in this laboratory, we have quantified the uptake rates of nitrogenous metabolites by intestine from arterial blood in vivo as well as the release rates of nitrogenous products into intestinal venous blood. The only nitrogen-containing metabolite extracted in large quantities was glutamine. The amounts of glutamine nitrogen taken up were balanced by the rates at which nitrogen was released as ammonia (38%), citrulline (28%), alanine (24%) and proline (7%).

Additional studies were then performed to assess the quantitative importance of this nitrogen-processing function of the intestine. The ammonia, alanine, and proline released by intestine are transported via the portal vein directly to the liver where all three are readily utilized as nitrogenous precursors for urea biosynthesis. The rate of hepatic urea biosynthesis was determined by measuring the urinary output of urea in fasted as well as non-fasted rats. The results show that in fasted animals about 30% of the urea produced in the liver is synthesized from nitrogenous precursors derived from the metabolism of circulating glutamine in the small intestine. In non-fasted animals, the rate of circulating glutamine metabolism by intestine

increases about 2-fold. In addition, the fed intestine metabolizes most of the dietary glutamine and all of the dietary aspartate and glutamate, yielding the same urea precursors derived from circulating glutamine. Our quantitative measurements reveal that in the fed animal 50-60% of hepatic urea synthesis utilizes nitrogenous substrates derived from the intestinal metabolism of dietary and circulating amino acids. Amino acids are clearly an important source of energy for the intestine; furthermore, the intestine is a site where the nitrogen derived from muscle and from certain dietary amino acids, toxic if absorbed in large quantities, is converted to forms utilized readily by the liver for conversion to urea, which is non-toxic and eliminated from the body by the kidneys.

Citrulline in the liver is an intermediate metabolite on the pathway leading to the biosynthesis of arginine and urea. From vascular perfusion studies in small intestine, we have shown that unlike in liver, citrulline in intestine is itself an end-product of nitrogen metabolism and undergoes no further metabolism. Additional studies with isolated perfused rat livers showed that this organ neither takes up the citrulline released by intestine nor does it release any citrulline of its own. Under physiological perfusion conditions, in which the liver was provided with ammonia and amino acids, all citrulline synthesized by the liver was further metabolized to arginine and then to urea, the only product released into the circulation.

Kidneys are another tissue with the necessary enzymes for converting citrulline to arginine, although the capacity of kidneys to synthesize citrulline is very low. We have developed a procedure for simultaneously sampling arterial and renal venous blood of rats, thus permitting precise arteriovenous difference measurements to be made across this organ. Using this technique, we have found a large uptake of circulating citrulline by the kidneys. In each blood pass through the kidneys, 35% of the citrulline is extracted. The rate of renal citrulline uptake, based on estimates of the renal blood flow, is approximately equal to the rate of intestinal citrulline release. Furthermore, on a molar basis, the kidneys release arginine into the blood at about 82% the rate of citrulline uptake. Since the high level of arginase in liver prevents the release of any arginine, the kidneys, utilizing citrulline produced by the intestine, appear to be the major source of circulating arginine, an amino acid needed for protein synthesis in all tissues.

(B) Role of small intestine in plasma apolipoprotein biosynthesis. Circulating lipoproteins are high-molecular weight complexes that serve as transport vehicles for various lipids among the body tissues. Elevated concentrations of certain plasma lipoproteins and depressed levels of others are associated with abnormal deposition of lipids in arterial endothelial cells (arteriosclerosis). We still know relatively few details regarding lipoprotein structure, synthesis, and metabolism, owing largely to the complex nature of these heterogenous particles. Rat plasma lipoproteins contain at least 10 different polypeptides or apolipoproteins, largely analogous to the constituent apolipoproteins of human plasma. Physiological functions have been deduced for some of these proteins -- in the assembly and transcellular trans-

port of these complexes and in their metabolism in blood and other tissues. The functions of some other of these proteins remain obscure.

In previous work from this and other laboratories, liver and intestine were identified as the only sources of plasma apolipoproteins. The pattern of hepatic apolipoprotein synthesis was determined in isolated, perfused rat livers. To determine the pattern of intestinal synthesis, we developed a preparation of autoperfused rat small intestine which permitted recovery of intestinal lymph, important because most intestinal lipoprotein products reach the blood via the lymph. Liver and intestine were found to have very different but overlapping patterns of apolipoprotein synthesis. Since several of the proteins were synthesized by both organs, two methods were developed to quantitate the relative contributions of each. In method 1, intestinal and hepatic products are differentially labeled by administering [^{3}H]leucine intraduodenally and [^{14}C]leucine intraportally to conscious rats. The $^{3}H/^{14}C$ ratios of individual plasma apolipoproteins are determined and related mathematically to reference proteins from liver (albumin) and intestine (selected apolipoproteins isolated from mesenteric lymph). In method 2, the patterns of apolipoprotein synthesis observed in isolated liver and in isolated intestine are related by multiple regression analysis to the pattern observed in the intact rat, where the contributions of both organs are reflected. Results with the two complementary methods were in good agreement and, when applied in fat-fed rats, showed that intestine contributed 50-60% of two of the major proteins of plasma high-density lipoproteins, namely apolipoproteins A-I and A-IV (apoA-I and apoA-IV). About 20% of the circulating apoB (major protein of low density lipoprotein) was also synthesized by intestine.

To determine if this unexpectedly large intestinal contribution of several apolipoproteins was related to the transport of dietary fat, the same methods have now been applied in two groups of rats not given fat. In one group, all dietary fat was witheld for 24 hours. In another group, a fat-free diet was supplied for 10-14 days, and in addition, hepatic bile was diverted from the intestine to also eliminate the major endogenous source of lipids in the intestine. The results indicate that while the absence of fat reduces the intestinal production of apoB, intestinal production of apoA-I and apoA-IV continue at about the same rates as during massive fat transport. The route by which these proteins enter the circulation is quantitatively altered, however. During fat transport, most of the newly-synthesized intestinal protein enters via mesenteric lymph in association with chylomicrons, large triacylglycerol-rich lipoprotein particles. In the absence of dietary or endogenous luminal lipid, more of these proteins enter intestinal venous blood directly, by-passing the lymph.

Preliminary results have also been obtained in rats made hypercholesterolemic by additions of cholesterol, taurocholate and propylthiouracil to the diet. In these animals, compared with normally fed controls, the rates of apoB synthesis relative to the other apolipoproteins appear to be increased. The increase appears due to a change in the pattern of apolipoprotein synthesis in the liver.

Significance of Biomedical Research and the Program of the Institute: These studies add to our understanding of intestinal, hepatic and renal function; in particular they help to define the roles of these organs in the biogenesis of circulating lipoproteins, in the metabolism of circulating and luminal substrates, and in the complex metabolic interrelationships that exist among the various body organs. In addition, they provide the methods and basic information for subsequent analyses of organ function under altered and pathological conditions.

Proposed Course: To further explore the role of the small intestine in lipoprotein formation, to further characterize the intestinal lipoprotein products both chemically and physiologically, and to compare the metabolism of lipoproteins of hepatic and intestinal origin. We also hope to further exploit the autoperfused intestinal preparation to answer questions regarding the transport, secretory, and metabolic activity of the small intestine.

Publications:

(1) Wu, A.-L. and Windmueller, H.G.: Relative contributions by liver and intestine to individual plasma apolipoproteins in the rat. J. Biol. Chem. 254: 7316-7322, 1979.

(2) Windmueller, H.G. and Spaeth, A.E.: Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats; quantitative importance of glutamine, glutamate, and aspartate. J. Biol. Chem. 255: 107-112, 1980.

(3) Windmueller, H.G. and Spaeth, A.E.: Vascular Perfusion of Rat Small Intestine for Permeation and Metabolism Studies. In Czaky, T.Z. (Ed.): Intestinal Permeation, Handbook of Experimental Pharmacology. New York, Springer-Verlag (in press).

(4) Windmueller, H.G.: Enterohepatic Aspects of Glutamine Metabolism. In Meister, A., Cohen, P.P. and Palacios, R. (Eds.): Glutamine: Metabolism, Enzymology, and Regulation. Mexico City, National University of Mexico Press (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-15400-06-LNE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Hormones, Lipoprotein Lipase and Lipid Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Scow	Chief, Section on Endocrinology	LNE, NIAMDD
	Sidney S. Chernick	Scientist Director	LNE, NIAMDD

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Nutrition and Endocrinology

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have used 3T3-L1 fibroblasts for studying hormonal regulation of lipoprotein lipase (LPL) in adipocytes. Earlier studies showed that insulin is most important in expression of the fat cell phenotype, development of LPL and deposition of triacylglycerol (TG). Insulin is also the primary regulatory factor of LPL in mature 3T3-L1 fat cells: It stimulates at once LPL activity. We recently found that antibodies to human insulin receptors duplicate effects of insulin on the enzyme in 3T3-L1 cells. 3T3-L1 fat cells also contain hormone-sensitive lipase, which hydrolyzes intracellular TG. Epinephrine and norepinehrine both stimulated release of glycerol from cells without affecting LPL activity. Although insulin treatment for 18 h stimulated LPL activity in these cells, it did not affect stimulation of glycerol release by catecholamines. The mechanisms for controlling these LPL and hormone-sensitive lipase appear to be separate and distinct. We are now producing antibodies to purified LPL of 3T3-L1 fat cells. The antibodies neutralize activity of the enzyme and will be used to study induction of LPL synthesis in 3T3-L1 fat cells.

Project Description:

Objectives

To determine the influence of hormones, lipoprotein lipase and other factors on the metabolism of lipid in adipose tissue, mammary gland, and other tissues. To determine how hormones regulate lipoprotein lipase activity in tissues. To develop a culture system for mammary and adipose cells that support differentiation of functions in response to hormones in vitro.

Methods Employed

Animals are deprived of endocrine glands and treated with hormones or other substances. Effects of the above on lipid metabolism and lipoprotein lipase activity in tissues are determined with in vivo and in vitro studies using conventional and radioisotopic techniques. Effects of hormones added in vitro are also studied. Cells isolated from adipose, mammary, and other tissues are grown in culture and used for study of mechanism of action of hormones.

Major Findings

Regulation of Lipoprotein Lipase and Hormone-sensitive Lipase in 3T3-L1 Fat Cells

During the past two years we have established that the mouse 3T3-L1 fibroblast cell line provides an excellent model system for studying hormonal regulation of lipoprotein lipase activity in adipocytes. Insulin was found to be the most important hormonal factor in expression of the fatty phenotype, development of lipoprotein lipase activity and deposition of triacylglycerol. In mature cells insulin has 3 distinct actions in the regulation of lipoprotein lipase: 1) It stimulates gene expression and RNA synthesis independently of effects on hexose and amino acid transport. 2) It stimulates hexose transport which is essential for expression of nuclear effects of insulin at the translational level. 3) It stimulates release of lipoprotein lipase from cells by a process that is independent of energy and uninhibited by colchicine and cytochalasin.

We recently found that antibodies to human insulin-receptors (ANti-R) mimicked the effects of insulin on lipoprotein lipase activity in mature 3T3-L1 fat cells. Anti-R also blocked the effects of insulin on the cells, suggesting that Anti-R and insulin both act through the same mechanism, and that metabolites of insulin are not "second messengers", internalized to act at nuclear sites, as proposed by others. (These findings were made in a collaborative study with Drs. E. Van Obberghen, C. R. Kahn, F. A. Karlsson, and C. Grunfeld of the Diabetes Branch of NIAMDD.)

We have also studied the regulation of hormone-sensitive lipase in 3T3-L1 fat cells. This lipase hydrolyzes intracellular triacylglycerol to fatty acids and glycerol for release by fat cells, whereas lipoprotein lipase acts on exogenous triacylglycerol to provide fatty acids for synthesis of triacylglycerol in the cells. Hormone-sensitive lipase is activated by catecholamines and other hormones acting through specific receptors and adenyl cyclase. Hormone-sensitive lipase was measured by the amount of glycerol released by cells to the medium. Epinephrine and norepinephrine

both stimulated hormone-sensitive lipase, but had very little effect on cellular lipoprotein lipase activity. Raising the molar ratio of fatty acids to albumin in the medium decreased hormone-sensitive lipase activity with minimal effect on lipoprotein lipase activity. Insulin and glucose enhanced both basal lipolysis and epinephrine-stimulated hormone-sensitive lipase activity. The results indicate that lipoprotein lipase and hormone-sensitive lipase are regulated independently in 3T3-L1 fat cells.

Antibodies to Lipoprotein Lipase of 3T3-L1 Fat Cells

Lipoprotein lipase released to incubation medium by 3T3-L1 cells in response to insulin has been purified by affinity-chromatography with heparin-sepharose. Antibodies to the purified enzyme have been raised in rabbits. The antibodies neutralize the activity of lipoprotein lipase and will be used to study the induction of enzyme synthesis and the roles of insulin and glucose in this process.

Significance to Biomedical Research and the Program of the Institute

Lipoprotein lipase is necessary for the uptake of triacylglycerol from blood by extrahepatic tissues. Earlier studies in this laboratory showed that the enzyme acts in capillary endothelium to hydrolyze triacylglycerol to partial glycerides and fatty acids which cross the capillary to be utilized by tissue cells. The present studies contribute information concerning the regulation and synthesis of lipoprotein lipase in adipocytes, the probable source of this enzyme in adipose tissue. It is evident that this enzyme plays a major role in the deposition of triacylglycerol in adipose tissue.

Proposed Course

Studies are in progress to produce specific antibodies to lipoprotein lipase from 3T3-L1 fat cells. The antibodies will be used to study synthesis of lipoprotein lipase in fat cells and the roles of insulin and glucose in this process. They will also be used to localize the enzyme in cells with immunocytochemical techniques and to study the mechanism of release of enzyme from the cells.

Publications

Spooner, P. M., Chernick, S. S., Garrison, M. M. and Scow, R. O.: Insulin regulation of lipoprotein lipase activity and release in 3T3-L1 adipocytes. J. Biol. Chem. 254: 10021-10029, 1979.

Van Obberghen, E., Spooner, P. M., Kahn, C. R., Chernick, S. S., Garrison, M. M., Karlsson, F. A., and Grunfeld, C.: Antibodies against the insulin receptor mimic a long-term insulin effect: Increase in lipoprotein lipase activity in 3T3-L1 fatty fibroblasts. Nature 200: 500-502, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-15401-08-LNE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Transport of Lipids, Hormones and Enzymes in Tissues, Cells and Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Scow	Chief, Section on Endocrinology	LNE, NIAMDD
	E. Joan Blanchette-Mackie	Research Biologist	LNE, NIAMDD
Other:	Mary G. Wetzel	Staff Fellow	LNE, NIAMDD

COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our group has developed a model for lipid transport between blood and tissue by lateral movement in a continuum of cell membranes. We recently tested this hypothesis in adipose tissue and heart from suckling rats. morphological findings in adipose tissue demonstrated continuity of intracellular and plasma membranes in endothelial and fat cells, and intercellular contacts between these cells. Biochemical studies showed that both chylomicron and cell triacylglycerol were hydrolyzed in glutaraldehyde-fixed adipose tissue when incubated at 25 C, and morphological studies showed that spiralling bilayered lamellae developed along the continuum between capillaries and fat cells. Morphometric analyses showed that lamellae developed first in capillaries and last in fat cells in adipose tissue from chylomicron-injected fed rats, whereas they developed first in fat cells and last in capillaries in tissue from fasted rats. We conclude lipolytic products formed in fixed tissues move laterally in a continuum of cell membranes to sites where they form lamellae as extensions of the continuum and thereby mark the route proposed for lipid transport between blood and tissue.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives

To study structure and metabolism of cells that synthesize and secrete lipids, hormones and enzymes. To study transport of lipids, hormones and enzymes across capillary endothelium, basement membrane and extracellular space in different tissues. To study uptake of lipids, hormones and enzymes by different cells. To study structure of cellular membranes and their role in lipid metabolism. To correlate structural with biochemical changes induced experimentally in cells and tissues. To study effects of lipolytic enzymes on lipid monolayers and lateral movement of products in the interface.

Methods Employed

Animals of different species and ages and in various physiological states (e.g., fasting, pregnant) are used to study with biochemical and morphological techniques synthesis, and storage or secretion of triacylglycerol in adipose tissue, heart, mammary gland, liver and intestines, and uptake and utilization of blood triacylglycerol by adipose tissue, heart, mammary gland and muscle. Morphology of cellular membranes and other structures are studied with the electron microscope and the freeze-fracture technique.

Monolayer techniques are used to study action of lipoprotein and other lipases on triacylglycerol and movement of lipolytic products in the gas-water interface.

Major Findings

Transport of Lipolytic Products from Blood to Adipose Tissue by Lateral Movement in a Continuum of Cell Membranes

Electron microscopic studies of adipose tissue from suckling rats were undertaken to explore the concept that lipids are transported from blood to tissue by lateral movement in a membrane continuum. Our aim was to obtain structural data showing membrane continuity between capillary endothelium and parenchymal cells, and to determine if lipids derived from lipolysis are associated with this continuum. We used developing white adipose tissue of suckling rats because adipocytes in this tissue are rapidly taking up fatty acids from chylomicron triacylglycerol derived from dietary (milk) lipid. To explore the structure of developing adipose tissue we used a variety of fixation and preparation techniques, including osmium tetroxide alone, osmium tetroxide and glutaraldehyde together, and glutaraldehyde followed by post-fixation in osmium tetroxide, and we found that they provided complimentary information. We also developed a technique to visualize cell to cell contacts and cell junctions using tannic acid (MW 1700). Tannic acid applied to adipose tissue after aldehyde fixation does not cross cell membranes and it reacts with osmium tetroxide to form an electron-opaque complex which is limited to the extracellular space. With this technique we were able to discover channels within adipocytes and capillary endothelium which are open to the extracellular space and also areas of cell to cell contact. The channels in endothelial cells extended between the luminal and basal surfaces,

and those in adipocytes extended from the cell surface to triacylglycerol droplets within the cells. Since these channels are open to the extracellular space, the membranes bounding them are continuous with the cell plasma membrane. Areas of contact between adipocytes excluded the tannic acid marker giving the appearance in sections of gap junctions. Areas of contact between capillary endothelium and adipocytes were also seen. In these areas basal projections of endothelial cells crossed through the underlying basement membrane material to adipocytes. Examination of cell membranes at these apposition areas (adipoendothelial junctions) showed that the external leaflets of adjacent plasma membranes came together to form a continuous leaflet. Our findings show that membrane continuity extends from the luminal surface of capillaries to the triacylglycerol droplet within adipocytes and that the external leaflets of cell membranes provide the interfacial continuum.

We further explored the possibility that lipids in transit between capillaries and adipocytes in developing white adipose tissue could be visualized with electron microscopic preparatory techniques. Previous studies on white adipose tissue of adult rats perfused with chylomicrons showed that hydrolysis of chylomicron triacylglycerol occurred after glutaraldehyde fixation when the tissue was incubated in buffer at 25 C. Electron microscopic studies on chylomicrons lipolyzed with lipoprotein lipase in vitro showed that amphipathic lipids could be seen as lamellar whorls with a banding pattern of 40 Å.

For our studies on lipolysis in developing white adipose tissue we used 8-12 day old rats, some of which were injected with chylomicrons and others were fasted overnight. Fat cells take up fatty acid from triacylglycerol in blood and release fatty acid from cell triacylglycerol during fasting. The former involves the action of lipoprotein lipase and the latter the action of tissue lipase within fat cells. Adipose tissue from the animals was fixed in glutaraldehyde and then incubated in buffer solution for varying periods of time. Tissue for biochemical analyses was taken after fixation and after incubation. Tissue for morphological analysis was osmicated either immediately after aldehyde fixation or after incubation.

Biochemical analyses showed that lipolysis of both chylomicron and cell triacylglycerol ocurred in glutaraldehyde-fixed tissue when incubated, and morphological findings in these tissues showed that spiralling bilayered lamellae developed along the continuum between capillaries and fat cells. Morphometric studies of electron micrographs showed that the amount of lamellae increased with incubation time and that location of lamellae varied with both incubation time and metabolic state of the tissue donor. In tissue of chylomicron-injected fed rats lamellae developed first in capillaries and last in fat cells, whereas in tissue of fasted rats lamellae developed first in fat cells, near triacylglycerol droplets, and last at the luminal surface of capillaries. Lamellae were not found in tissue of chylomicron-injected fed rats if the tissue was incubated with insulin and glucose prior to fixation, indicating that the lamellae were composed of lipolytic products (fatty acid and monoacylglycerol). Thus, in glutaraldehyde-fixed tissue from chylomicron-injected fed rats the lamellae represent accumulations of lipolytic products moving along the continuum during incubation from capillary lumina to

fat cells, whereas in tissue from fasted rats they represent lipolytic products moving from fat cells to capillary lumina. We conclude that amphipathic lipids are transported in adipose tissue by lateral movement in a continuum of cell membranes. (E. J. Blanchette-Mackie and R. O. Scow)

Morphological Studies of Lipolysis and Lipid Transport in Rat Heart

The ultrastructure of hearts of 12-14 day old rats injected with chylomicrons was studied to test in this tissue our model for lipid transport from blood to tissue by lateral movement in a continuum of cell membranes. Initial surveys showed progressive accumulation of lamellar structures with incubation at 25 C in tissues previously fixed with glutaraldehyde. Biochemical analyses showed that chylomicron-triacylglycerol was hydrolyzed and that the products formed, fatty acids, accumulated in glutaraldehyde-fixed hearts when incubated at 25 C. These findings indicated that lipoprotein lipase activity was preserved in fixed tissues and that the lamellar whorls probably represented accumulations of amphipathic lipolytic products. Electron micrographs of tissue were analyzed morphometrically to obtain data concerning location and amount of lamellar structures present in tissues under various conditions. The largest number of lamellae developed in tissues of fed rats injected with chylomicrons, while the smallest number developed in tissues of uninjected fasted rats. The lamellae were found along the proposed route of transport, in capillary lumina, within channels in capillary endothelial cells, in intercellular spaces, and within channels in ventricular muscle cells. Muscle cells of hearts from uninjected fed rats contained numerous lipid droplets, usually associated with mitochondria. Although the amount of lipid in cells was reduced in hearts from rats fasted overnight, lipid droplets were still found in the tissue. Thus, accumulation of lamellae in hearts of uninjected rats, either fed or fasted, could result from hydrolysis of cell triacylglycerol. In such cases, lamellae were found near mitochondria, a primary site of fatty acid utilization in heart. Lamellar whorls did not develop in heart tissue immediately fixed in osmium tetroxide after being removed from the animal. These findings demonstrate that chylomicron-triacylglycerol and also cell triacylglycerol can be hydrolyzed in glutaraldehyde-fixed heart, and that the products formed move laterally in an interfacial continuum of cell membranes to sites where they form lamellar whorls as extensions of the continuum. We conclude that lipolytic products are transported in heart tissue by lateral movement in a continuum of cell membranes. (M. G. Wetzel and R. O. Scow)

In vitro Lipolysis of Human Chylomicrons by Lipoprotein Lipase

A study of the effect of lipoprotein lipase on human chylomicrons in vitro was carried out in collaboration with Drs. E. Schaefer and B. Brewer of NHLBI and Drs. G. Bengtsson and T. Olivecrona of Umeå, Sweden. Chylomicrons obtained from cannulated thoracic ducts of normolipidemic patients fasted overnight were incubated with purified bovine milk lipoprotein lipase in albumin/Tris solution, pH 7.4, at 37 C for 60 min. Biochemical, immunological and morphological analyses were performed on lipoprotein fractions of various densities resulting from lipolysis of chylomicrons. Apoproteins of chylomicrons prelabeled with radioiodine distributed primarily to the d 1.019-1.063 (g/ml), d 1.063-1.21 and d >1.21 fractions after >90% hydrolysis of chylomicron triacylglycerol. Acyl glycerol, cholesterol and

apoprotein B were found predominantly in d 1.019-1.063 (LDL) fraction and apoproteins A-I and A-II in the d 1.063-1.21 (HDL) and d >1.21 fractions, while apoprotein C-III was fairly evenly distributed between the three more dense fractions, d >1.019.

Lipoprotein particles in the various fractions were negatively stained with phosphotungsate and examined with the electron microscope. The findings, which confirmed our earlier observations on rat chylomicrons, showed progressive changes in configuration of chylomicrons undergoing lipolysis and appearance of remnants of surface film associated with variable amounts of core material. The resultant fragments were found in the denser lipoprotein fractions (d >1.019), but they did not resemble the HDL particles found in corresponding fractions of normal human plasma; they were generally larger and more irregular in outline. We conclude they are fragments of surface components (proteins, phospholipid and cholesterol) from lipolyzed chylomicrons. (M. G. Wetzel and R. O. Scow)

Significance to Biomedical Research and the Program of the Institute

Uptake of triacylglycerol from chylomicrons and very low density lipoproteins (VLDL) requires hydrolysis of triacylglycerol to fatty acid and monoacylglycerol by lipoprotein lipase acting at the luminal surface of the capillary endothelium. Our earlier studies on clearance of chylomicrons from blood led to the development of a model for transport of lipid from blood to tissue by lateral movement in a continuous interface composed of cell membranes. Our findings reported above provide structural evidence for an interfacial continuum of membranes extending from the luminal surface of capillaries to the endoplasmic reticulum of parenchymal cells. A similar route is probably used for transport of other amphipathic substances (e.g., cholesterol, phospholipids, certain amino acids, steroid hormones) from blood to tissue cells and transport of fatty acids within cells from lipid stores to mitochondria. Abnormal accumulation of lipid in blood vessels and other tissues probably involves lipid transport by lateral movement in cell membranes.

Proposed Course

The action of lipoprotein lipase on chylomicrons in capillaries and transport of lipids between and within cells will be studied in heart, mammary gland and brown fat with biochemical and morphological techniques, including freeze-fracture. Small intestines of suckling rats will be studied with the electron microscope to determine if lipid digestion and absorption involves lipid transport by lateral movement in cell membranes. The role of lipoprotein lipase in the formation of high density lipoproteins (HDL) from chylomicrons will be studied in vitro. The lipid monolayer technique will be used to study action of various lipases on triacylglycerol and phospholipids, and movement of different lipids in the interfacial plane.

Publications

Berendsen, P. B., and Blanchette-Mackie, E. J.: Milk lipid absorption and chylomicron production in the suckling rat. Anat. Rec. 195: 397-413, 1979.

Scow, R. O., Blanchette-Mackie, E. J., and Smith, L. C.: Transport of lipid across capillary endothelium. Fed. Proc. 39: #9, 1980.

Scow, R. O., Desnuelle, P., and Verger, R.: Lipolysis and lipid movement in a membrane model: Action of lipoprotein lipase. J. Biol. Chem. 254: 6456-6463, 1979.

Smith, L. C., and Scow, R. O.: Intravascular Metabolism of Lipoproteins: Chylomicrons. Mechanism of Transfer of the Lipolytic Products to Cells. In Paoletti, R. and Eisenberg, S. (Eds.): Progress in Biochemical Pharmacology: Lipoprotein Metabolism. Basel, Karger, A. G., 1979, pp. 109-138.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-15402-05-LNE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Pharyngeal Lipase and Digestion of Dietary Fat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Scow	Chief, Section on Endocrinology	LNE, NIAMDD
Other:	Ruth B. Field	Guest Worker	LNE, NIAMDD

COOPERATING UNITS (if any)
None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pharyngeal tissues in man and rat secrete a lipase which acts in the stomach, at pH 5.4, to hydrolyze triacylglycerol to the amphipathic products diacylglycerol, monoacylglycerol and fatty acids. This first step in digestion of dietary fat facilitates emulsification of triacylglycerol. The objectives of the project are: 1) isolate and purify pharyngeal lipase from tissues of rats and other species and from pharyngeal secretions of man, 2) characterize the mode of action of pharyngeal lipase, 3) study regulation and secretion of the enzyme, and 4) determine its role in fat digestion in both normal and diseased states in infants and adults. Experiments to isolate pharyngeal lipase from serous glands of rat tongue show ten-fold purification when lipolytic activity in the 850 x g supernatant of tissue homogenates is precipitated with $(NH_4)_2SO_4$ and desalted with Sephadex G-25. Lipolytic activity in the extract is stable at -20 C for several months. Assay for lipolytic activity has been improved by using an artificial emulsion of trioleoylglycerol as substrate and incubating in medium containing Ca^{2+} and taurodeoxycholate instead of albumin.

Project Description:

Objectives

To purify pharyngeal lipase from tissues of animals and from pharyngeal secretion of man. To characterize the structure and mode of action of the enzyme. To study regulation and secretion of the enzyme. To determine the role of pharyngeal lipase in fat digestion.

Methods Employed

Lingual serous glands from rats and other species, and esophageal secretions from man are used as sources of pharyngeal lipase. Extracts containing lipolytic activity are fractionated by various techniques in order to isolate and purify the enzyme. The mechanism of action of the enzyme on triacylglycerol is studied using artificial emulsions, chylomicrons and lipid monolayers.

Major Findings

We have started a program to isolate pharyngeal lipase from serous glands of rat tongues. It was previously determined that pH 5.4 is optimal for lipolytic activity of the enzyme, measuring the amount of fatty acids released from triacylglycerol. The substrate used in the current experiments was an emulsion of trioleoylglycerol and tri[9.10-^{3}H]oleoylglycerol, stabilized with phosphatidylcholine, in glycerol. The assay procedure has been improved by incubating in medium containing 3.34 mM $CaCl_2$ and 17 mM taurodeoxycholate, instead of albumin. We have modified Belfrage-Vaughan's technique for extracting fatty acids from incubation medium by using 0.2 M glycine-NaOH, pH 12.4, for the aqueous phase. This assures a pH of 11.0-11.5 in the upper phase and a consistent, maximal partitioning of fatty acids, 75-80%, into the upper phase.

The rate of lipolytic activity in extracts of lingual serous glands was linear for 2 min when assayed in medium containing 5.6 µmol of trioleoylglycerol and the amount of fatty acids produced was between 50 and 500 nmol. There was delay in onset of maximal activity when less than 50 nmol were formed, and partial suppression of activity when more than 500 nmol were formed. The mechanisms of these effects will be studied.

Homogenates of rat lingual serous glands were centrifuged at 850 x g and the supernatant used for isolating pharyngeal lipase. Preliminary studies had shown that separation of microsomes from this fraction did not increase enzyme activity/mg protein in either the microsomal fraction or the 106,000 x g supernatant. A ten-fold purification was obtained by precipitating with $(NH_4)_2SO_4$ and then desalting with Sephadex G-25. Filtration through Sephadex G-200 resulted in 96% recovery of protein and only 20% recovery of lipolytic activity in the void volume when eluted with 0.0125 M Tris-maleate buffer, pH 5.4. There was no increase in activity when the other fractions were added to the protein fraction. Much greater loss of protein and activity occurred when pH of the eluent was 7.4. We are now testing hydrophobic chromatography for use in isolating and purifying pharyngeal lipase.

Significance to Biomedical Research and the Program of the Institute

It is now widely accepted that pharyngeal lipase accounts for the lipolytic activity found in gastric contents, and that the products formed by this enzyme facilitate emulsification of triacylglycerol before it enters the intentines. The role of this enzymic reaction in patients with abnormal fat digestion and in premature babies has not been studied.

Proposed Course

Isolation and purification of pharyngeal lipase from lingual tissues of rats will be continued. When pure preparations of the lipase are available, studies will be started concerning the mode of action of the lipase on various lipids.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-15403-02-LNE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Diabetes, Insulin and Blood Vessels

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Scow	Chief, Section on Endocrinology	LNE, NIAMDD
Other:	Sidney S. Chernick	Scientist Director	LNE, NIAMDD

COOPERATING UNITS (if any)
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is presently inactive.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15500-20 LNE

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Large-scale Processing of Biological Materials

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Joseph Shiloach Head, Pilot Plant LNE NIAMDD

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SUMMARY OF WORK (200 words or less - underline keywords)

The Pilot Plant has been involved in the production of large quantities of microorganisms and mammalian cells for the isolation and purification of biologically important macromolecules and cell particulates and to assist NIH investigators in the scale-up and processing of plant and animal tissues for biological substances such as enzymes, vitamins, alkaloids and hormones.

Project Description

Objectives: To provide facilities and services for the processing and/or production of large amounts of natural materials, such as bacteria, yeasts, molds, protozoa, animal cells, blood, urine and plant and animal tissues, so that they may be studied under standard laboratory conditions.

Methods Employed: To satisfy the large-scale production needs of NIH investigators, methods are designed for the scaling up of small laboratory experiments utilizing large reaction vessels, extractors, stills, concentrators, centrifuges, etc., so that the processing can be carried out effectively, efficiently and safely. Three 10-liter, one 50-liter, one 100-liter, one 300-liter and one 1100-liter fermentors are utilized for growing microorganisms and animal cells under a variety of conditions. Bacterial cells and yeasts are harvested by means of laboratory or large-scale AS-16P and AS-26P Sharples centrifuges. Protozoa are concentrated in low speed centrigures. Cells are either supplied as a paste or futher processed to yield cell-free suspensions by rupture with a Gaulin homogenizer. In some instances, a specific substance is isolated and partially purified by further processing of the culture supernatant or the cell paste using conventional fractionation procedures and the newer chromatographic techniques.

Major Findings: During the past 10 months, a total of 136 requests were processed for investigators: NIAMDD 52; BOB32; NCI 25, NHLBI 14; NICHD 3; NIAID 7, NIMH 1; and NEI 2. Organisms produced include wild type and mutant strains of Aspergillus fumigatus, Bacillus subtilis, Bordetella pertussis, Escherichia coli, Euglena, Haemophilus influenzae, Neisseria meningitides, Neurospora, Pseudomonas putida, Saccharomyces cerevisiae, Salmonella typhimurium, Staphlococcus aureus, and Tetrahymena pyriformis. Human lymphocytes, mouse lymphoma cells and Chinese Hamster Ovary cells were also grown in fermentors. Some of the microorganisms were used for the production of Bacteriophages T4, T7 and lambda; others were used to produce extracellular and intracellular enzymes.

Studies were continued with the Division of Bacterial Products, Bureau of Biologics for polysaccharide production by H. influenzae and Streptococcus and isolation of the enterotoxin proteins from special strains of E. coli. Sphereoplasts were produced for the NHLBI from E. coli using 10-, 50-, and 300-liter fermentors in a series. Crude interferon fractions were isolated from cultures of transformed beta-lymphocytes stimulated with Newcastle Disease virus, strain B-1.

Large scale processing activities consisted of the isolation of enzymes such as aldolase reductase from human placentae, phosphodiesterase from bovine brain; and adenyl succinase from yeast. Calmodulin was purified from 33 lbs. of bovine testicles and protein A was isolated from Staplococcus aureus. Hormones and growth factors were isolated from various microorganisms and erythrocyte ghosts were prepared for enzyme subunit purification. Two-gallon quantities of hydroxyapatite were prepared in the 50-gallon Pfaudler glass-lined reactor. Various plant and animal tissues were dried and/or ground in the

large oven, mills and related equipment and solutions were centrifuged, concentrated or lyophilized.

Proposed Course of Project: The Pilot Plant hopes to better control growth of microorganisms and mammalian cells by monitoring technqiues developed within the last year. The requirements of cell growth will be observed to increase the yields of cells and their products. New computer technology for optimization of cell growth will be explored. New technologies for fermentations and separations will be employed.

Publications: None

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

A. Studies On A Mammalian Hepatic Binding Protein Specific For Asialoglycoproteins

1. Evidence for the recycling of the hepatic receptor for asialoglycoproteins was obtained in isolated rat hepatocytes. Freshly prepared hepatocytes attained maximal ability to bind, internalize and degrade ^{125}I-asialo-orosomucoid after 5 hours in suspension culture of 37°. Comparison of the number and distribution of the binding sites revealed that 12.5×10^5 receptors were present, per cell, on the external cell membrane and that approximately ten times this number were located intracellularly. Binding was time-dependent, saturable, and dissociable. The dissociation constant for the single high affinity binding site was calculated to be 3.4×10^{-8}M. Binding and uptake studies in the presence of cyclohexamide indicated that at least 17 times more ligand was metabolized than could be bound by the cell surface receptors. These results provide convincing evidence for receptor recycling, or re-utilization, in the functionally intact hepatocyte.

2. Continuing studies on the topological distribution of the receptor for asialoglycoproteins have demonstrated the cytosolic surface of the hepatic plasma membrane to be devoid of ligand binding activity. This finding excluded the possibility that inverted vesicles, formed during the preparation of subcellular organelles, could be responsible for the cytosolic binding observed earlier in the lysosome-enriched fraction. An alternate approach employed the formation of ferritin-labeled antibody complexes with the lysosomal constituents. Subsequent election microscopic examination of this material was inconclusive and led to the realization that the hepatic binding protein extended through the lipid bilayer of the membrane and was exposed on the cytosolic surface. The potential role of this fragment in signaling receptor-occupancy is under investigation.

3. Attempts to insert the specifically labeled receptor into hepatocytes have been undertaken in an effort to monitor subcellular events during the endocytosis of ligand. Initial studies have shown that the solubilized hepatic binding protein associates spontaneously with small unilamellar lipid vesicles. This process is independent of the phase transition of the lipid and effectively restores the specific binding activity of the receptor. Circular dichroism studies indicated that the protein undergoes a conformational change on association with lipid. Binding of specific ligand produces further physical changes in the receptor as indicated by alterations in the tryptophan fluoresce quenching pattern.

B. The Formation of the Yeast Primary Septum.

1. Studies on the localization of chitin in the yeast cell wall are being extended by examining, with the electron microscope, the localization of chitin in yeast thin sections which had been labeled with wheat germ agglutin-colloidal gold complexes. Initial results have confirmed

the feasibility of this approach. It is hoped to use this procedure to determine the distribution of chitin in very early stages of bud emergence. This information is essential for an understanding of the mechanism of localized initiation of chitin synthesis in vivo. In an effort to attack the problem from a different point of view, experiments have begun to investigate the ability of cell cycle temperature-sensitive mutants to accumulate chitin when the cell cycle is interrupted by shifting to the non-permissive temperature. Preliminary results show a preferential accumulation of chitin in mutants blocked in early, but not late portions of the cycle. Work is continuing on this topic, coupled with attempts to determine the location of the accumulated chitin with the ultrastructural procedures outlined above.

2. Activation of the chitin synthetase zymogen.

The finding of yeast mutants lacking proteinase B, the putative activator of chitin synthetase zymogen, prompted a search for an alternative mechanism of activation of the enzyme. It was found that incubation of intact cells of proteinase B-negative mutant with 1% digitonin led to an activation of chitin synthetase that could be partially inhibited by the protease inhibitor antipain or by EDTA. An extract obtained by treating intact cells with 0.1% digitonin and mercaptoethanol is able to activate, in a time-dependent fashion, a solubilized chitin synthetase zymogen. This process, however, is not inhibited by antipain. The thermolabile factor present in the extract is now being purified.

3. An endochitinase from yeast.

During a previous study on the effect of a wheat germ chitinase on nascent chitin made by yeast chitin synthetase, it was found that the synthetase was contaminated with chitinase activity. It seemed important to study further this activity, both because of its ability to interfere with chitin synthetase determinations, and for its possible physiological significance. The chitinase has now been purified by adsorption on chitin, release from the chitin by autodigestion and chromatography on SP-Sephadex. After electrophoresis of the purified preparation on polyacrylamide gels a strong reaction for carbohydrates was found in coincidence with the enzymatic activity, an indication that the chitinase may be a glycoprotein. Characterization of the activity, which is still in progress, showed it to be of the endo type, i.e. the reaction products are oligosaccharides.

Perhaps the most interesting finding regards the intracellular distribution of the enzyme. The highest specific activity was found in the vacuole plasmic space. Therefore, the enzyme seems to be destined for secretion. It may have a role in cell separation, the last step of the cell cycle, in which partial hydrolysis of the cell wall is probably needed to set free mother and daughter cell.

4. The biosynthesis of β(1→3)glucan, the major polysaccharide of the yeast cell wall.

An activator of glucan synthetase present in boiled yeast extracts has now been identified as GTP. Several attempts to solubilize the

synthetase with a variety of detergents were unsuccessful. These attempts are continuing. Further studies on the kinetics of the enzyme were also carried out. A new family of potent inhibitors of the synthetase was found. These compounds are double-headed trinucleotides of the type ApppU, ApppG and GpppG, where both nucleoside moieties are linked to phosphate at the 5' position. Experiments with these compounds as well as with structural analogs of ATP suggest that the latter nucleotide may stimulate the synthetase by direct binding, in addition to participating in an enzymatic modification of the system. It also appears that the two activators, ATP and GTP, are binding to different sites.

C. Hormone Dependent Differentiation of Mammary Gland.

1. Estrogen (E_2) has long been recognized as one of the hormones required for the proliferation of mammary epithelial cells in vivo. The steroid has not been previously implicated, however, in the differentiation of these cells. In fact, the cells in mammary explants from intact virgin animals can differentiate (i.e., make casein and α-lactalbumin) in the absence of exogenous E_2 as long as insulin (I), glucocorticoid (F) and prolactin (P) are added to the medium. Nevertheless, it has been possible to demonstrate that exogenous E_2 can enhance the formation of milk proteins in the presence of the other hormones and T_3. Is E_2 an absolute requirement for such induction? Yes, but long-term ovariectomized mice must be used as the source of mammary tissue in order to establish this requirement. Under these conditions a lesion, the inability of mammary epithelium to synthesize the milk proteins in the presence of I, F and P, develops. The lesion can be prevented and corrected by administration of E_2; it is not related to a deficiency of prolactin. The studies reveal an additional dimension in the relationship between E_2 and mammary development.

2. As indicated above, I, F and P are required for the formation of casein and α-lactalbumin in mammary explants from intact virgin mice. P has been considered the primary hormonal inducer, and I and F regarded as ancillary agents. Recent observations, using mammary tissue from pregnant rats, assign a more fundamental role to I and F. These two hormones can induce α-lactalbumin activity in this tissue in the absence of exogenous P. Kinetic studies indicate that such induction is not a reflection of retention of endogenous P. Neither casein nor fatty acids are induced under these conditions. Progesterone administration in vivo make it possible for mammary explants from virgin rats to respond to I and F in regard to α-lactalbumin activity. The results reveal qualitative differences in hormonal requirements of various milk constituents, and suggest that progesterone may play a heretofore unsuspected role in mammary differentiation.

D. Polyamines in the Hormone-Dependent Development of Mammary Gland.

1. Synergistic actions of insulin, glucocorticoid and prolactin induce proliferation of the epithelial cells and formation of the milk proteins casein and alpha-lactalbumin in organ culture of mouse mammary gland. During the course of investigation of the mechanisms involved in the hormonal regulation of milk protein synthesis, immunochemical method to determine the synthesis of casein and alpha-lactalbumin as well as a quantitative assay method to measure the amount of alpha-lactalbumin have

been developed following the isolation and purification of the two milk proteins. Using these methods, it was found that the synthesis of casein and alpha-lactalbumin are not synchronous and subject to the differential, concentration-dependent action of cortisol: the optimal concentrations of cortisol required for maximal induction of casein and alpha-lactalbumin are 3×10^{-6}M and 3×10^{-8}M, respectively. Moreover, 10^{-7} to 10^{-5}M cortisol caused progressive inhibition of alpha-lactalbumin accumulation. By the use of optimal culture conditions for the induction of the two milk proteins, cyclic AMP has been shown to act as a negative regulator of hormonally induced lactogenesis in mammary cells.

2. The hormonal stimulation of polyamine biosynthesis in cultured mammary explants is mediated by increases in the activity if various enzymes in the polyamine biosynthetic pathway. The activity of ornithine decarboxylase, which catalyzes the formation of putrescine from ornithine, increases in a biphasic manner. The first peak of activity is caused largely by incubation of tissue explants in a hormone-free medium and is sensitive to the osmolarity of the culture medium. Experiments employing various culture conditions in which the intracellular level of putrescine was varied by alterations in the osmolarity of culture medium, use of several inhibitors of ornithine decarboxylase, and use of mammary explants containing varying amounts of endogenous level of putrescine indicate that an early rise in the level of ornithine decarboxylase and putrescine is essential for hormonal stimulation of DNA synthesis.

E. Differentiation of Lymphoid Cells.

1. In earlier studies it was shown that the lipid A moiety of lipopolysaccharide induced the enhanced secretion of non-immunoglobulin proteins by bone marrow cells but not by any of the many other types of cells tested. This novel phenotypic expression of lipopolysaccharide responsiveness was assessed for bone marrow cells derived from lipopolysaccharide-responder, non-responder and -low-responder mouse strains and found to correlate only partially with the lipopolysaccharide responsiveness assessment based solely on the mitogenic reactivity of splenocytes. These findings indicate that the phenotype based on the lipopolysaccharide enhanced secretion of non-immunoglobulin protein will be useful in characterizing more precisely the polysaccharide response in regard to the many biological activities that are known to be affected by lipid A although controlled at a single genetic locus in responder/nonresponder mice.

A simple method has been devised using a single glass surface for the purification, identification and autoradiographic analysis of lipid A binding by individual macrophage cells derived from spleen and peritoneal fluids. The finding that individual macrophage cells varied in their ability to bind ^{125}I-lipid A is consistent with the growing body of data indicating the heterogeneity of macrophage cells. This general method is also amenable to other kinds of studies in which the interactions between radiolabeled compounds and individual macrophage cells are of specific interest and should be useful, as well as, indicating possible differentiation antigens whose expression may vary with the maturity of individual macrophage cells within a given population.

2. In continuation of a study of the mitogenic activity of nucleotides like ATP toward T cells, the extent of ATP stimulation of cell proliferation was examined by simultaneously studying the kinetics of DNA synthesis and kinetics of appearance of radioactivity in individual cells using autoradiography. The data indicate that proliferation is limited and appears to be restricted to a single round of cells duplication unlike the multiple rounds of cell duplication that have been ordinarily observed with other mitogenic agents such as concanavalin A, lectins and lipopolysaccharide. ATP, rendered insoluble via covalent linkage to agarose, was as effective as soluble ATP when used at one-tenth the normal concentration. It is likely because of steric considerations involved in bringing agarose particles and cells in contact, that insoluble ATP may be equally as effective as soluble ATP at even lower concentrations.

F. Enzymatic Basis of Detoxication

Prior work with the sulfotransferases had ascribed a number of catalytic activities to specific enzymes; the literature contains the names of a dozen or so different sulfotransferases that include an N-hydroxy-2-acetylaminofluorene sulfotransferase, phenol sulfotransferase, tyrosine methylester sulfotransferase, cortisol sulfotransferase, and dehydroepiandrosterone sulfotransferase, among others. As a result of the availability of sulfotransferases in homogeneous form, it has been possible to assess the substrate specificity of these enzymes and to approach their mechanism of action.

It is now clear that the enzymes found active with a variety of hydroxysteroids are actually a single group having broad specificity for primary and secondary alcohols. Three hydroxysteroid sulfotransferases appear to have the same substrate specificity, including similar kinetic constants, despite the different chemical and physical composition of the proteins. Aside from the hydroxysteroids and the series of aliphatic alcohols beginning with ethanol, sulfate esters are formed with ascorbic acid, propranolol and chloromycetin.

The phenol (aryl) sulfotransferases fall into two groups because only one group is able to catalyze the sulfation of tyrosine methylester, organic hydroxylamines or epinephrine; both groups of enzymes are active with a large number of other phenols. At present, the kinetics of the phenol sulfotransferase reaction is undergoing study.

G. Biochemical abnormalities underlying human cystinosis

1. The abnormal accumulations of free cystine in both cystinotic and I-cell fibroblasts have been found to be localized almost exclusively in the lysosomal fractions of these cells.

2. The elevated cystine content of both types of cultured cells can be markedly reduced by incubation of the cells in media containing low concentrations of the disulfide-reducing agent cysteamine.

fold to the native state. However, the present studies show that with pepsinogen, this is the last step in the folding sequence and that the protein can develop most of its secondary structure before this rate limiting step.

If pepsinogen is exposed to diethyl pyrocarbonate (DEP) while undergoing this slow folding reaction, one of the three histidine residues in the protein reacts readily with this reagent. In the case of the fully folded protein the reaction is very slow. Pepsinogen which has been modified in this way shows reduced potential pepsin activity (0-30%) suggesting the involvement of this region of the molecule in the zymogen's intramolecular activation to pepsin. Analysis of activation mixtures shows the presence of fully active pepsin, present in reduced yield (0-30%) and potential pepsin activity is not restored by prolonged exposure to hydroxylamine which reverses the DEP-histidine reaction. This suggests that modification of the zymogen is occurring at other positions which would not be detected spectrophotometrically. ^{14}C-labeled DEP has been synthesized and reacted with pepsinogen as above. A total of 1.9 moles of label are incorporated, 0.9 mole of which is not released by hydroxylamine. The modified protein has been digested with trypsin and four labeled peptides have been obtained, suggesting that inactivation results from partial reactions with other residues in the molecule.

J. Enzymes of Carbohydrate Metabolism in invertebrates and vertebrates

1. Two major ATP:hexose 6-phosphotransferases (hexokinases) have been identified in tissues of Homarus americanus (lobster) and separated from each other by DEAE-cellulose ion exchange chromatography and by polyacrylamide gel electrophoresis. The molecular weight of each, determined by gel filtration, is about 50,000. Hexokinase II, named for its column elution order, resembles hexokinase isozymes I and II of vertebrates. K_m values for glucose, mannose and fructose are 0.08, 0.13 and 6.7 mM, respectively. It is strongly inhibited by the reaction products, ADP and glucose-6-P (K_i = 0.8 mM). Hexokinase I appears to be different from any animal hexokinase previously described. It has a high affinity for mannose and fructose and low affinity for glucose.

K. Properties and mechanism of action of L-myoinositol-1-phosphate

1. Based on the fact, among other, that NAD^+ is required for the reaction the pathway between glucose 6-phosphate and inositol 1-phosphate is postulated to involve 5-ketoglucose 6-phosphate and inosose-2 1-phosphate. After dephosphorylation of all reaction products of borotritide reduction and addition and re-isolation of carrier D-glucitol, L-iditol, myoinositol, and scylloinositol all but iditol were found labeled. The labeling of the two cyclitols clearly establishes inosose-2 1-phosphate as an intermediate. The significance of the iditol result cannot be evaluated until flucitol is degraded to ascertain whether the 5-position, overwhelmed by the label in position 1, is labeled.

Gamma globulin from serum of rabbits immunized against homogeneous rat testis inositol 1-phosphate synthase contained antibody specifically cross-reactive with the same enzyme and with crude preparations of testis,

3. Incubation of cystinotic cells at somewhat elevated temperatures (e.g. 39-40°C) results in marked diminution of the cystine content in comparison with control cells grown at 35-36°; in contrast, similar incubation of I-cells at the same elevated temperatures is without effect on the cystine content. Conversely, at temperatures lower than normal (e.g. 25°C), the cystine content of cystinotic cells increases somewhat in comparison with cells grown at the normal temperature, whereas similar incubation of I-cells at 25° results in significantly depressed levels of intracellular free cystine.

4. Incubation of I-cell fibroblasts in media containing secreted protein from normal fibroblasts results in marked diminution of intracellular cystine; in contrast, there is little or no change in the cystine content of cystinotic fibroblasts when incubated under identical conditions in the presence of the same secretions.

H. Studies on naturally occurring sulfur nucleotides

1. The new E. coli mutant absolutely deficient in s^4U in its tRNA should be very useful in investigating the two-step thiolation reaction. Toward this end, single tRNA species lacking s^4U have been purified from this strain for use as sulfur-acceptors. Two-dimensional TLC procedures have been set up to identify the site(s) of thiolation after the chains are incubated with the proper enzymes. Light may also be thrown on the intermediate in the two-step reaction.

2. The λ-produced tRNA thiopurine resists identification. It is not 7-methyl-6-thioguanine, which had previously been a possibility. Although it can be isolated by HPLC, it dethiolates and disintegrates rapidly, and identification by mass spectroscopy failed.

The modified tRNA species is one which is overproduced after λ-induction, as judged by gel electrophoresis. Fingerprint patterns exist for all these species, although all species are not yet paired with amino acids. In the absence of amino acid accepting activity in the newly thiolated species, fingerprint studies have been undertaken. The sulfur-modified oligonucleotide may also be more amendable to identification of the thionucleotide.

3. A second λ mutant carrying an amber mutation in the site of the thiolating enzyme has been studied. In this case too, the amber mutation has been suppressed by insertion of tyrosine, but not of serine or glutamic acid, at the amber site.

I. The folding of swine pepsinogen from the randomly coiled form to its native structure has been shown to be a complex reaction with at least two detectable intermediates. The slowest step in this reaction sequence has a rate which is essentially independent of pH and varies with temperature with $H^{\ddagger} = 20 \pm 2$ kcals mole^{-1}. These are characteristics of the cis-trans isomerization of peptide bonds adjacent to proline residues, which other workers have suggested occurs in the unfolded form of all proteins. Only those molecules which have peptide bonds in the correct configurations can

epidiymis, brain, and ovary by Ouchterlony double diffusion. In testing for cross-reactivity by precipitation both brain and testis immunoprecipitates were found to retain 60% of the enzymatic activity of the soluble enzyme. In one application of the immunochemical method it became feasible to assay the synthase at early stages of purifiction under conditions which precluded the use of conventional methods used in later stages. An overall purification of 450-fold between testis homogenate and pure enzyme was achieved.

L. Biochemical lesions in genetic mucopolysaccharides

1. A novel sulfohydrolase, specific for the 3-0 sulfate ester of methyl-2-deoxy-2-sulfamino-α-D-glucopyransoside 3-sulfate, has been purified approximately 70-fold from human urine. By means of definitively synthesized ^{35}S-labeled glucosamine derivatives, it has been established that the enzyme a) is specific for the 3-0 sulfate ester and b) requires that the adjacent amino group be sulfated rather than free or acetylated.

2. A disulfated glucosamine residue corresponding to the synthetic enzyme substrate is not known to exist in any natural substance. Among the glycosaminoglycans, only heparin, and to a lesser extent, heparan sulfate, can readily accommodate a 3-0 sulfated glucosulfamate moiety. It was therefore proposed that one or more such residues might be present in heparin and plays a key role in its physiological interaction with antithrobin. Collaborative studies with Dr. Ulf Lindahl have confirmed this hypothesis. Using the purified 3-0 sulfatase as a structural probe, evidence has been obtained indicating that both 3,6-disulfated glucosulfamate residues, resistant to the sulfatase, and 3-0 sulfated residues susceptible to enzymatic hydrolysis, are uniquely present in heparin fragments having a high affinity for antithrombin.

M. The Function of the Bacterial Outer Membrane Components.

1. Interaction of three outer membrane components to form a receptor. Using a variety of selection techniques (antibiotics, colicins, bacteriophages), ompF mutants have been isolated. Outer membrane material, prepared from these mutants, was missing detectable amounts of the OmpF protein. These mutants were completely resistant to colicin A and it was shown that they lack the outer membrane receptor for this colicin. Purified OmpF protein, in the presence of added lipopolysaccharide, was shown to inactivate colicin A. This inactivation was stimulated by added btuB gene product. The OmpF protein is an essential part of the colicin A receptor and at least 3 outer membrane components (the OmpF protein, the btuB gene product, and lipopolysaccharide) interact to form the active colicin A receptor. This appears to be an excellent model system for a study of how different membrane proteins interact to give the membrane its properties.

2. Chemical and immunological relationship of peptidoglycan-associated proteins. Peptidoglycan-associated proteins play an important role in the passive diffusion of hydrophilic substances across the outer membrane. Several of these proteins have been purified and labeled with radioactive iodine. Following proteolytic digestion, comparison of peptide

maps of different proteins showed the presence of several common hydrophobic peptides. The hydrophilic peptides were largely unique to each protein. These data are consistent with the notion that the hydrophobic region of the peptide, which is presumably buried within the membrane, is conserved. The immunological characterization of these proteins was complicated by the presence of a common outer membrane protein in the purified proteins used for immunization.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17001-14 LBM

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October 1, 1979 to September 30, 1980

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The Role of Hepatic Plasma Membranes in the Turnover of Circulating Glycoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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7	7	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Evidence for recycling of the hepatic membrane receptor for asialoglycoproteins has been obtained in freshly isolated rat liver hepatocytes. The topological distribution of the receptor in the plasma membrane has been examined. Functional binding activity is restricted to the outer surface of the membrane although the peptide chain penetrates the lipid bilayer and is exposed on the cytosolic surface. Insertion of the solubilized receptor into unilamellar lipid vesicles is accompanied by conformational changes associated with restoration of binding activity.

PHS-6040

Project Description:

(1) Evidence for the recycling of the hepatic receptor for asialoglycoproteins was obtained in isolated rat hepatocytes. Freshly prepared hepatocytes attained maximal ability to bind, internalize and degrade ^{125}I-asialo-orosomucoid after 5 hours in suspension culture at 37°. Comparison of the number and distribution of the binding sites revealed that 12.5×10^4 receptors were present, per cell, on the external cell membrane and that approximately ten times this number were located intracellularly. Binding was time-dependant, saturable, and dissociable. The dissociation constant for the single high affinity binding site was calculated to be 3.4×10^{-9}M. Binding and uptake studies in the presence of cyclohexamide indicated that at least 17 times more ligand was metabolized than could be bound by the cell surface recpetors. These results provide convincing evidence for receptor recycling, or re-utilization, in the functionally intact hepatocyte.

(2) Continuing studies on the topological distribution of the receptor for asialoglycoproteins have demonstrated the cytosolic surface of the hepatic plasma membrane to be devoid of ligand binding activity. This finding excluded the possibility that inverted vesicles, formed during the preparation of subcellular organelles, could be responsible for the cytosolic binding observed earlier in the lysosome-enriched fraction. An alternate approach employed the formation of ferritin-labeled antibody complexes with the lysosomal constituents. Subsequent electron microscopic examination of this material was inconclusive and led to the realization that the hepatic binding protein extended through the lipid bilayer of the membrane and was exposed on the cytosolic surface. The potential role of this fragment in signaling receptor-occupancy is under investigation.

(3) Attempts to insert the specifically labeled receptor into hepatocytes have been undertaken in an effort to monitor sub-cellular events during the endocytosis of ligand. Initial studies have shown that the solubilized hepatic binding protein associates spontaneously with small unilamellar lipid vesicles. This process is independent of the phase transition of the lipid and effectively restores the specific binding activity of the receptor. Circular dichroism studies indicated that the protein undergoes a conformational change on association with lipid. Binding of specific ligand produces further physical changes in the receptor as indicated by alterations in the tryptophan fluoresce quenching pattern.

(4) Significant progress has been made in the isolation and purification of the hepatic receptor for the haptoglobin-hemoglobin complex although the receptor is still far from homogeneity. Alternate methods of purification, including the preparation of monoclonal antibodies, are in progress.

Proposed Course of Project:

(1) It is proposed to investigate the significance of the transmembrane portion of the binding protein in signaling receptor-occupancy prior to endocytosis.

(2) Further identification of the vesicles in the lysosomal fraction which bear cytosolic-oriented binding sites will be sought.

(3) Alternate methodologies will be employed to insert the purified, radioactive receptor into the plasma membrane of viable hepatocytes.

(4) An attempt will be made to determine whether receptor-recycling involves mixing with the vastly greater pool of sub-cellular receptor.

(5) Isolation and purification of the haptoglobin-hemoglobin to homogeneity is an immediate goal.

Publications:

Steer, C. J., and Ashwell, G.: Studies on a mammalian hepatic binding protein specific for asialoglycoproteins: Evidence for receptor recycling in isolated rat hepatocytes. J. Biol. Chem. 255: 3008-3013, 1980.

Hubbard, A. L., Wilson, G., Ashwell, G., and Stukenbrok, M.: An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. J. Cell Biol. 83: 47-64, 1979.

Neufeld, E. F., and Ashwell, G.: Carbohydrate Recognition Systems for Receptor-Mediated Pinocytosis. In Lennarz, W.J. (Ed.): The Biochemistry of Glycoproteins and Proteoglycans. New York, Plenum Publishing Corp., 1980, pp. 241-266.

Klausner, R. D., Bridges, K., Tsunoo, H., Blumenthal, R., Weinstein, J. N., and Ashwell, G.: Reconstitution of the hepatic asialoglycoprotein receptor upon interaction with phospholipid vesicles. Proc. Natl. Acad. Sci. USA (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17002-10 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Enzymatic Basis of Detoxication

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William B. Jakoby	Chief, Section on Enzymes and Cellular Biochemistry	LBM	NIAMDD
OTHER:	Ellen Sue Lyon	Staff Fellow	LBM	NIAMDD
	Michael W. Duffel	Staff Fellow	LBM	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Recent work has led us to the perception of the enzymes of detoxication as a coherent group of about thirty or so catalytic activities with specific features in common. Primarily, these enzymes generally have a broad spectrum of substrates with specificity for a functional group rather than for the carbon skeleton. The substrates are generally lipophilic and a usual feature is a relatively low catalytic efficiency. Emphasis in this laboratory has been on enzymes active in conjugation reactions such as those catalyzed by glutathione transferases, thiol S-methyltransferases, and steroid and phenol sulfotransferases. Work with the sulfotransferases is allowing characterization of six homogeneous enzymes active in the formation of sulfate esters of phenols, primary and secondary alcohols including hydroxysteroids, and organic hydroxylamines.

Project Description:

Prior work with the sulfotransferases had ascribed a number of catalytic activities to specific enzymes; the literature contains the names of a dozen or so different sulfotransferases that include an N-hydroxy-2-acetylaminofluorene sulfotransferase, phenol sulfotransferase, tyrosine methylester sulfotransferase, cortisol sulfotransferase, and dehydroepiandrosterone sulfotransferase, among others. As a result of the availability of sulfotransferases in homogeneous form, it has been possible to assess the substrate specificity of these enzymes and to approach their mechanism of action.

It is now clear that the enzymes found active with a variety of hydroxysteroids are actually a single group having broad specificity for primary and secondary alcohols. Three hydroxysteroid sulfotransferases appear to have the same substrate specificity, including similar kinetic constants, despite the different chemical and physical composition of the proteins. Aside from the hydroxysteroids and the series of aliphatic alcohols beginning with ethanol, sulfate esters are formed with ascorbic acid, propranolol and chloromycetin.

The phenol (aryl) sulfotransferases fall into two groups because only one group is able to catalyze the sulfation of tyrosine methylester, organic hydroxylamines or epinephrine; both groups of enzymes are active with a large number of other phenols. At present, the kinetics of the phenol sulfotransferase reaction is undergoing study.

Proposed Course - Work with the conjugating enzymes of liver is intended to delineate the means by which we are able to survive in the presence of the many noxious and toxic compounds to which we are exposed. The investigation of the above noted projects will be continued with particular emphasis on the enzymes responsible for group transfer.

Publications:

Weisiger, R. A., and Jakoby, W. B.: Thiol S-methyltransferase from rat liver. Arch. Biochem. Biophys. 196: 631-637, 1979.

Sekura, R., and Jakoby, W. B.: Phenol sulfotransferases. J. Biol. Chem. 254: 5658-5663, 1979.

Weisiger, R. A., Pinkus, C. M., and Jakoby, W. B.: Potential role of thiol-S-methylsulfotransferase in the detoxication of intestinal hydrogen sulfide and methane thiol. Gastroenterology 76: 1261, 1979.

Lyon, E. S., and Jakoby, W. B.: The identity of alcohol sulfotransferase with hydroxysteroid sulfotransferase. Arch. Biochem. Biophys. 202: 474-481, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17003-13 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Polysaccharides in Morphogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Enrico Cabib	Research Chemist	LBM NIAMDD
OTHER:	Julieta Correa	Guest Worker	LBM NIAMDD
	Rowena Roberts	Staff Fellow	LBM NIAMDD
	Narayanasamy Elango	Visiting Fellow	LBM NIAMDD

COOPERATING UNITS (if any)

Blair Bowers, LCB, NHLBI

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Laboratory of Biochemistry and Metabolism

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several temperature-sensitive cell cycle mutants of Saccharomyces cerevisiae have been found to accumulate chitin at the restrictive temperature. Extracts from a proteinase B negative mutant, derived from the same organism, catalyze the activation of chitin synthetase zymogen. An endochitinase was purified from yeast and partially characterized. The enzyme appears to be preferentially localized in the vacuole fraction.

An activator of β(1→3)glucan synthetase found in boiled yeast extracts was purified and identified as GTP. A family of nucleotides containing two bases and three phosphates were found to be potent inhibitors of glucan synthetase.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The ongoing research attempts to provide an understanding of the molecular mechanisms of morphogenesis. The topics currently under study are the formation of the primary septum of yeast and the biosynthesis of a glucan which is a major structural component of the yeast cell wall.

Methods Employed: The organism used is Saccharomyces cerevisiae, grown in the laboratory. Cells are converted into protoplasts by treatment with snail intestinal juice. Protoplasts are disrupted by osmotic lysis and mechanical shearing to obtain particulate fractions. The latter are further fractionated by centrifugation in density gradients. Enzymes are solubilized with detergents and purified by conventional methods including affinity chromatography on appropriate adsorbents.

Wheat germ agglutinin and purified preparations of different chitinases are attached to colloidal gold, to obtain markers for the localization of chitin in cell walls and septa by electron microscopy. Adducts of the same proteins with fluorescein isothiocyanate are used as chitin markers for fluorescence microscopy.

Major Findings:

I. The formation of the yeast primary septum.

A) Localization of chitin in the yeast cell wall.

A paper on the localization of chitin in septa and cell wall, based on a study outlined in last year's report, has been published in the Journal of Cell Biology. These studies are now being extended by examining, with the electron microscope, the localization of chitin in yeast thin sections which had been labeled with wheat germ agglutinin-colloidal gold complexes. Initial results have confirmed the feasibility of this approach. It is hoped to use this procedure to determine the distribution of chitin in very early stages of bud emergence. This information is essential for an understanding of the mechanism of localized initiation of chitin synthesis in vivo. In an effort to attack this problem from a different point of view, experiments have begun to investigate the ability of cell cycle temperature-sensitive mutants to accumulate chitin when the cell cycle is interrupted by shifting to the non-permissive temperature. Preliminary results show a preferential accumulation of chitin in mutants blocked in early, but not late portions of the cycle. Work is continuing on this topic, coupled with attempts to determine the location of the accumulated chitin with the ultrastructural procedures outlined above.

B) Activation of the chitin synthetase zymogen.

The finding of yeast mutants lacking proteinase B, the putative activator of chitin synthetase zymogen, prompted a search for an alternative

mechanism of activation of the enzyme. It was found that incubation of intact cells of proteinase B-negative mutant with 1% digitonin led to an activation of chitin synthetase that could be partially inhibited by the protease inhibitor antipain or by EDTA. An extract obtained by treating intact cells with 0.1% digitonin and mercaptoethanol is able to activate, in a time-dependent fashion, a solubilized chitin synthetase zymogen. This process, however, is not inhibited by antipain. The thermolabile factor present in the extract is now being purified.

C) An endochitinase from yeast.

During a previous study on the effect of a wheat germ chitinase on nascent chitin made by yeast chitin synthetase, it was found that the synthetase was contaminated with chitinase activity. It seemed important to study further this activity, both because of its ability to interfere with chitin synthetase determinations, and for its possible physiological significance. The chitinase has now been purified by adsorption on chitin, release from the chitin by autodigestion and chromatography on SP-Sephadex. After electrophoresis of the purified preparation on polyacrylamide gels a strong reaction for carbohydrates was found in coincidence with the enzymatic activity, an indication that the chitinase may be a glycoprotein. Characterization of the activity, which is still in progress, showed it to be of the endo type, i.e. the reaction products are oligosaccharides.

Perhaps the most interesting finding regards the intracellular distribution of the enzyme. The highest specific activity was found in the vacuole fraction. There are indications that part of the enzyme may be in the periplasmic space. Therefore, the enzyme seems to be destined to secretion. It may have a role in cell separation, the last step of the cell cycle, in which partial hydrolysis of the cell wall is probably needed to set free mother and daughter cell.

II. The biosynthesis of β(1→3)glucan, the major polysaccharide of the yeast cell wall.

Two papers were recently published in the Journal of Biological Chemistry on this topic.

An activator of glucan synthetase present in boiled yeast extracts has now been identified as GTP. A poster presentation of this finding will be made at the 5th International Symposium on Yeasts, in London, Ontario, Canada.

Several attempts to solubilize the synthetase with a variety of detergents were unsuccessful. These attempts are continuing. Further studies on the kinetics of the enzyme were also carried out. A new family of potent inhibitors of the synthetase was found. These compounds are double-headed trinucleotides of the type ApppU, ApppG and GpppG, where both nucleoside moieties are linked to phosphate at the 5' position. Experiments with these compounds as well as with structural analogs of ATP suggest that the latter nucleotide may stimulate

the synthetase by direct binding, in addition to participating in an enzymatic modification of the system. It also appears that the two activators, ATP and GTP, are binding to different sites.

Publications

Shematek, E. M., Braatz, J. A., and Cabib, E.: Biosynthesis of the yeast cell wall. I. Preparation and properties of β-(1→3)glucan synthetase. J. Biol. Chem. 255: 888-894, 1980.

Shematek, E. M., and Cabib, E.: Biosynthesis of the yeast cell wall. II. Regulation of β-(1→3)glucan synthetase by ATP and GTP. J. Biol. Chem. 255: 895-902, 1980.

Molano, J., Bowers, B., and Cabib, E.: Distribution of chitin in the yeast cell wall. An ultrastructural and chemical study. J. Cell Biol. 85: 199-212, 1980.

Cabib, E., Duran, A., and Bowers, B.: Localized Activation of Chitin Synthetase in the Initiation of Yeast Septum Formation. In Burnett, J.H. and Trinci, A.P.J. (Eds.): Fungal Walls and Hyphal Growth. Cambridge, Cambridge University Press, 1979, pp. 189-201.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 17004-12 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Peter McPhie Research Chemist LBM NIAMDD

OTHER: Fang-Ting Chou Visiting Fellow LBM NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD, LBM, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	-

SUMMARY OF WORK (200 words or less - underline keywords)

Our work is directed towards the relationship of protein sequence to conformation and enzymic activity. The digestive enzyme, pepsin, is synthesized as an inactive precursor pepsinogen. We are interested in the molecular basis of the proteolytic activity of the enzyme, how this is suppressed in the zymogen and is induced during activation.

PHS-6040

Project Description:

Methods - Visible and Ultraviolet spectroscopy: Optical rotatory dispersion and circular dichroism; Viscosity; Enzyme Assays; Rapid kinetic techniques using stopped flow and T-jump apparatus.

Major Findings -

The folding of swine pepsinogen from the randomly coiled form to its native structure has been shown to be a complex reaction with at least two detectable intermediates. The slowest step in this reaction sequence has a rate which is essentially independent of pH and varies with temperature with $\Delta H^{\ddagger} = 20 = 2$ kcals mole^{-1}. These are characteristics of the cis-trans-isomerization of peptide bonds adjacent to proline residues, which other workers have suggested occurs in the unfolded form of all proteins. Only those molecules which have peptide bonds in the correct configurations can fold to the native state. However, the present studies show that with pepsinogen, this is the last step in the folding sequence and that the protein can develop most of its secondary structure before this rate limiting step.

If pepsinogen is exposed to diethyl pyrocarbonate (DEP) while undergoing this slow folding reaction, one of the three histidine residues in the protein reacts readily with this reagent. In the case of the fully folded protein the reaction is very slow. Pepsinogen which has been modified in this way shows reduced potential pepsin activity (0-30%) suggesting the involvement of this region of the molecule in the zymogen's intramolecular activation to pepsin. Analysis of activation mixtures shows the presence of fully active pepsin, present in reduced yield (0-30%) and potential pepsin activity is not restored by prolonged exposure to hydroxylamine which reverses the DEP-histidine reaction. This suggests that modification of the zymogen is occurring at other positions which would not be detected spectrophotometrically. ^{14}C-labeled DEP has been synthesized and reacted with pepsinogen as above. A total of 1.9 moles of label are incorporated, 0.9 mole of which is not released by hydroxylamine. The modified protein has been digested with trypsin and four labeled peptides have been obtained, suggesting that inactivation results from partial reactions with other residues in the molecule.

Proposed Course -

The present study will be completed by purification of these peptides in increased yield and their identification by amino acid analysis and sequence determination.

Mechanistic studies on pepsin show that, unlike most other enzymes, reactions with "good" substrates are characterized by greatly increased k_{cat} values, whereas K_m's are low and invariant. This has been interpreted to show that a conformational change in the protein is very important in

the peptidase reaction. A number of derivatives of pepsin have been prepared which have spectrophotometric or fluorometric reporter groups close to the active site. Since these show only slightly reduced enzymic activity these can be used to study the behavior of the protein during the reaction.

Publications:

McPhie, P.: The alkaline transition of swine pepsinogen. Biophys. Chem. 9: 281-287, 1979.

Ahmad, F. and McPhie, P.: Characterization of a stable intermediate in the unfolding of diazoacetylglycine ethyl ester-pepsin by Urea. Can. J. Biochem. 57: 1090-1092, 1980.

McPhie, P.: Kinetic studies on the unfolding and refolding of pepsinogen in urea. The nature of the rate-limiting step. J. Biol. Chem. 255: 4048-4052, 1980.

Ahmad, F. and McPhie, P.: The intrinsic viscosity of glycoproteins. Int. J. Biochem. 11: 91-96, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17005-15 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of Naturally Occurring Sulfur Nucleotides: Identification, Chemistry, Biosynthesis and Biological Significance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marie N. Lipsett	Research Chemist	LBM	NIAMDD
OTHER:	Sankar L. Adhya	Res. Microbiologist	LMBG	NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, BGS, NCI

LAB/BRANCH
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A second E. coli mutant lacking 4-thiouridine (s^4U) in its tRNA has been identified. The absolute deficiency arises from the lack of the first of two thiolating enzymes. The pattern of the other tRNA thionucleotides is normal. Some s^4U-free tRNA species have been purified for further use.

The unidentified thiopurine produced after bacteriophage λ infection of E. coli occurs in a species of tRNA overproduced on induction. This tRNA species thus far accepts no amino acid. Two different amber mutations in the thiolation region of λ are relieved by tyrosine- but not by glutamic acid- or serine-inserting suppressors.

PHS-6040

Project Description:

Major Findings and Proposed Course of Work:

1. The new E. coli mutant absolutely deficient in s^4U in its tRNA should be very useful in investigating the two-step thiolation reaction. Toward this end, single tRNA species lacking s^4U have been purified from this strain for use as sulfur-acceptors. Two-dimensional TLC procedures have been set up to identify the site(s) of thiolation after the chains are incubated with the proper enzymes. Light may also be thrown on the intermediate in the two-step reaction.

2. The λ-produced tRNA thiopurine resists identification. It is not 7-methyl-6-thioguanine, which had previously been a possibility. Although it can be isolated by HPLC, it dethiolates and disintegrates rapidly, and identification by mass spectroscopy failed.

The modified tRNA species is one which is overproduced after λ induction, as judged by gel electrophoresis. Fingerprint patterns exist for all these species, although all species are not yet paired with amino acids. In the absence of amino acid accepting activity in the newly thiolated species, I have decided to identify it by fingerprint. The sulfur-modified oligonucleotide may also be more amenable to identification of the thionucleotide.

3. In collaboration with Dr. S. Adhya, a second λ mutant carrying an amber mutation in the site of the thiolating enzyme has been studied. In this case too, the amber mutation has been suppressed by insertion of tyrosine, but not of serine or glutamic acid, at the amber site.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17006-06 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Function of Bacterial Outer Membrane Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John D. Foulds	Research Chemist	LBM	NIAMDD
OTHER:	Joyce Sturgeon	Staff Fellow	LBM	NIAMDD

COOPERATING UNITS (if any)
John Robbins, NIH, Bureau of Biologics
Mike Zasloff, NIH, GBB/NIAMDD
Hiroshi Nikaido, University of California, Berkeley

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NIAMDD, LEM, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interaction of a minor protein component of the Escherichia coli outer membrane with a major porin protein to form an active colicin receptor has been demonstrated. This may represent a common mechanism whereby minor protein components of the outer membrane (each with a specific transport function) interact with major proteins (each with a non-specific transport function) to accomplish selected functions. These functions for example, include promoting the facilitated diffusion of hydrophilic substances such as vitamin B12 or ferrichrome across the outer membrane.

PHS-6040

Project Description:

Objectives - The goal of this project is to describe the role of individual components (proteins, phospholipids, lipopolysaccharide) of the Escherichia coli outer membrane in the function of this membrane.

(1) To examine the properties and components of the outer membrane receptor for vitamin B12

(2) To determine the relationship among distinct major outer membrane proteins which serve to promote the diffusion of Phydrophilic substances across the outer membrane

(3) To examine the interaction of outer membrane proteins which have "specific" transport functions with proteins which have "non-specific" transport functions

Major Findings -

A. Interaction of three outer membrane components to form a receptor. Using a variety of selection techniques (antibiotics, colicins, bacteriophages), ompF mutants have been isolated. Outer membrane material, prepared from these mutants, was missing detectable amounts of the OmpF protein. These mutants were completely resistant to colicin A and it was shown that they lack the outer membrane receptor for this colicin. Purified OmpF protein, in the presence of added lipopolysaccharide, was shown to inactivate colicin A. This inactivation was stimulated by added btuB gene product. The OmpF protein is an essential part of the colicin A receptor and at least 3 outer membrane components (the OmpF protein, the btuB gene product, and lipopolysaccharide) interact to form the active colicin A receptor. This appears to be an excellent model system for a study of how different membrane proteins interact to give the membrane its properties.

B. Chemical and immunological relationship of peptidoglycan-associated proteins. Peptidoglycan-associated proteins play an important role in the passive diffusion of hydrophilic substances across the outer membrane. Several of these proteins have been purified and labeled with radioactive iodine. Following proteolytic digestion, comparison of peptide maps of different proteins showed the presence of several common hydrophobic peptides. The hydrophilic peptides were largely unique to each protein. These data are consistent with the notion that the hydrophobic region of the peptide, which is presumably buried within the membrane, is conserved. The immunological characterization of these proteins was complicated by the presence of a common outer membrane protein in the purified proteins used for immunization.

C. Collaborative work -

1. John Robbins (Bureau of Biologics). The E. coli K-1 antigen is often associated with pathogenecity. The genetic information for the production of the K-1 antigen has been placed in a laboratory K-12 strains to facilitate studies on the effect on pathogenecity of mutations which alter cell surface components.

2. H. Nikaido (University of California). The properties of the pore formed by protein E have been compared with those formed by other outer membrane proteins.

3. Mike Zasloff (GBB/NIAMDD). The protein composition of cell envelopes of isogenic rough and smooth strains of Pseudomonas have been compared. Although significant, reproducible differences were not seen, these studies will be refined to examined only those proteins associated with the outer membrane.

Proposed Course -

1. The vitamin B12 uptake system. Loss of a specific outer membrane protein results in the loss of the E. coli outer membrane receptor which participates in the energy-independent phase of vitamin B12 uptake. This protein also participates in the formation of another, distinct receptor for colicin A. The individual components will be purified and an attempt will be made to reconstitute these receptors in vitro.

2. Specific and non-specific diffusion pores. The outer membrane contains a variety of specific receptors which participate in the diffusion of selected substances across the membrane. In each case a specific outer membrane protein is associated with this function. Some of these proteins will be purified and an attempt made to determine if their function depends upon the concommitant participation of other outer membrane components such as porin-proteins or lipopolysaccharide.

Publications

Chai, T., and Foulds, J.: Isolation and partial characterization of protein E, a major protein found in certain Escherichia coli K-12 mutant strains. J. Bacteriol. 139: 418-423, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18000-15 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Hormone-Dependent Development of Mammary Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yale J. Topper	Chief, Section on Intermediary Metabolism	LBM	NIAMDD
OTHER:	Lakshmanan Sankaran	Senior Staff Fellow	LBM	NIAMDD
	Kevin Nicholas	Visiting Fellow	LBM	NIAMDD
	Kapalavayi Nagaiah	Visiting Associate	LBM	NIAMDD
	Franklyn Bolander, Jr.	Research Associate	LBM	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	5.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Estrogen is an absolute requirement for the differentiative potential of mouse mammary gland.

Although insulin, glucocorticoid and prolactin are required for induction of casein and fatty acids in mammary explants from pregnant rats, only insulin and glucocorticoid appear necessary for induction of α-lactalbumin activity in this tissue.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives - To study the molecular and cytological phenomena involved in the development of the mammary gland. This includes attempts to understand the mechanism of action of insulin, prolactin, glucocorticoids, estrogen, progesterone and thyroid hormone on this tissue.

Methods Employed - Mammary gland explants, mammary epithelial cells or mammary cell nuclei are cultured in synthetic media. Development of α-lactalbumin activity and lactose synthetase activities are determined by enzymatic means. Synthesis of casein is determined immunochemically, or by electrophoretic isolation of ^{32}P-labeled phosphoproteins. Casein mRNA is assayed by cDNA hybridization. DNA and RNA syntheses are studied using conventional methods.

Major Findings -

A. Estrogen (E_2) has long been recognized as one of the hormones required for the proliferation of mammary epithelial cells in vivo. The steroid has not been previously implicated, however, in the differentiation of these cells. In fact, the cells in mammary explants from intact virgin animals can differentiate (i.e., make casein and α-lactalbumin) in the absence of exogenous E_2 as long as insulin (I), glucocorticoid (F) and prolactin (P) are added to the medium. Nevertheless, it has been possible to demonstrate that exogenous E_2 can enhance the formation of milk proteins in the presence of the other hormones and T_3. Is E_2 an absolute requirement for such induction? Yes, but long-term ovariectomized mice must be used as the source of mammary tissue in order to establish this requirement. Under these conditions a lesion, the inability of mammary epithelium to synthesize the milk proteins in the presence of I, F and P, develops. The lesion can be prevented and corrected by administration of E_2; it is not related to a deficiency of prolactin. The studies reveal an additional dimension in the relationship between E_2 and mammary development.

B. As indicated above, I, F and P are required for the formation of casein and α-lactalbumin in mammary explants from intact virgin mice. P has been considered the primary hormonal inducer, and I and F regarded as ancillary agents. Recent observations, using mammary tissue from pregnant rats, assign a more fundamental role to I and F. These two hormones can induce α-lactalbumin activity in this tissue in the absence of exogenous P. Kinetic studies indicate that such induction is not a reflection of retention of endogenous P. Neither casein nor fatty acids are induced under these conditions. Progesterone administration in vivo makes it possible for mammary explants from virgin rats to respond to I and F in regard to α-lactalbumin activity. The results reveal qualitative differences in hormonal requirements of various milk constituents, and suggest that progesterone may play a heretofore unsuspected role in mammary differentiation.

Proposed Course of Project -

1. We will attempt to determine the molecular basis for the essentiality of estrogen in relation to mammary differentiation.

2. The roles of insulin, glucocorticoid and prolactin in the induction of casein mRNA will be studied.

3. Hormonal requirements for DNA synthesis by isolated mammary cell nuclei will be investigated.

Significance to Biomedical Research and the Program of the Institute - Hormones are involved in normal growth and differentiation, and are implicated in many disease states. The mammary gland system provides an excellent opportunity to increase our understanding about the ways in which a number of different types of hormones interact with target cells.

Publications

Topper, Y. J., and Freeman, C. S.: Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev. (in press).

Bolander, F. F., Jr., and Topper, Y. J.: Stimulation of lactose synthetase activity and casein synthesis in mouse mammary explants by estradiol. Endocrinology 106: 490-495, 1980.

Bolander, F. F., Jr., Nicholas, K. R., and Topper, Y. J.: Retention of glucocorticoid by isolated mammary tissue may complicate interpretation of results from in vitro experiments. Biochem. Biophys. Res. Comm. 91: 247-252, 1979.

Bolander, F. F., Jr., and Topper, Y. J.: Relationships between spermidine, glucocorticoid and milk proteins in different mammalian species. Biochem. Biophys. Res. Comm. 90: 1131-1135, (1979).

Nicholas, K. R., and Topper, Y. J.: Enhancement of α-lactalbumin-like activity in mammary explants from pregnant rats in the absence of exogenous prolactin. Biochem. Biophys. Res. Comm. (in press).

Bolander, F. F., Jr., and Topper, Y. J.: Loss of differentiative potential of the mammary gland in ovariectomized mice. Endocrinology (in press).

Bolander, F. F., Jr., and Topper, Y. J.: Estrogen in Relation to the Synthesis of Milk-Protein. In Rich, M. and McGrath, C. (Eds.): Systematics of Mammary Cell Transformation. New York, Academic Press (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 18001-06 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Role of Free and Lipid-bound Inositol in Brain and Cell Membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Frank Eisenberg, Jr.	Research Chemist	LBM NIAMDD
OTHER:	Toshio Maeda	Visiting Fellow	LBM NIAMDD
	Ryuichi Hasegawa	Visiting Fellow	LBM NIAMDD

COOPERATING UNITS (if any)

None

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SECTION
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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Further properties and the mechanism of action of homogeneous inositol 1-phosphate synthase (EC 5.5.1.4) from rat testis cytosol have been examined. Inosose-2 1-phosphate has been shown indirectly, but conclusively, to be the cyclic intermediate which is reduced presumably by NADH to the product. Antibody to the enzyme prepared in rabbits cross reacts with all rat tissue synthases studied. The immunoprecipitated enzyme is 60% as active as the soluble enzyme. Overall purification of the enzyme, described previously, has now been determined at 450-fold with introduction of the immunochemical assay.

PHS-6040
(Rev. 10-76)

Project Description:

Objective - Properties and mechanism of action of L-myoinositol 1-phosphate synthase.

Methods - L-myoinositol 1-phosphate synthase (EC 5.5.1.4) was isolated from rat testis cytosol and purified to homogeneity as described previously. In studying reaction mechanism the enzyme was incubated with glucose 6-phosphate and NAD^+ and after 30 sec NaB^3H_4 was added and intermediates sought by addition of putative carriers which were then re-isolated and examined for radioactivity. In preparing antibody homogeneous enzyme was injected intracutaneously in rabbits and gamma globulin was isolated from the serum by ammonium sulfate precipitation. The protein was tested for antibody activity by precipitation with crude and pure synthases from various sources and by Ouchterlony double diffusion.

Major Findings - Based on the fact, among others, that NAD^+ is required for the reaction the pathway between glucose 6-phosphate and inositol 1-phosphate is postulated to involve 5-ketoglucose 6-phosphate and inosose-2 1-phosphate. After dephosphorylation of all reaction products of borotritide reduction and addition and re-isolation of carrier D-glucitol, L-iditol, myoinositol, and scylloinositol all but iditol were found labeled. The labeling of the two cyclitols clearly establishes inosose-2 1-phosphate as an intermediate. The significance of the iditol result can not be evaluated until glucitol is degraded to ascertain whether the 5-position, overwhelmed by the label in position 1, is labeled.

Gamma globulin from serum of rabbits immunized against homogeneous rat testis inositol 1-phosphate synthase contained antibody specifically cross-reactive with the same enzyme and with crude preparations of testis, epididymis, brain, and ovary by Ouchterlony double diffusion. In testing for cross-reactivity by precipitation both brain and testis immunoprecipitates were found to retain 60% of the enzymatic activity of the soluble enzyme. In one application of the immunochemical method it became feasible to assay the synthase at early stages of purification under conditions which precluded the use of conventional methods used in later stages. An overall purification of 450-fold between testis homogenate and pure enzyme was achieved.

Significance - Inositol 1-phosphate synthase accounts for all de novo synthesis of myoinositol, the principal monomeric sugar of the brain. Of the nine possible isomeric inositols, several of which occur in nature, only the myo isomer is the precursor of phosphatidylinositol, the phospholipid of membrane uniquely responsive to extracellular stimuli manifested by increased turnover of inositol and phosphate. The inositol phospholipids, moreover, are the principal contributors of arachidonic acid, the precursor of prostacyclins and thromboxanes, the proper balance of which assures the normal function of certain components of the circulatory system.

Proposed Course - Further work currently underway will be done to identify intermediates in the reaction. The antibody-enzyme interaction is being studied in further application to assay, localization, and purification of the enzyme in other tissues. Hormonal effects on the development of the enzyme in vivo are being investigated. The structure of the enzyme will be examined further with a view toward defining the role of subunits.

Publications:

Maeda, T., and Eisenberg, F., Jr.: Purification, structure, and catalytic properties of L-myoinositol 1-phosphate synthase from rat testis. J. Biol. Chem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER
-		Z01 AM 18002-07 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Investigations on the Pathogenesis of Human Cystinosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Frank Tietze Research Chemist LBM NIAMDD

COOPERATING UNITS (if any)

Dr. Joseph D. Schulman, Dr. J. D. Butler, Dr. R. Steinhertz: Section on Human Biochemical and Developmental Genetics, NPMD, NICHHD.

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1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cultured skin fibroblasts from patients with I-cell disease exhibit abnormally high intralysosomal levels of free cystine and in this respect are strikingly reminiscent of the characteristic property of cultured cells from individuals affected with the unrelated genetic disorder cystinosis. To determine whether the abnormal cystine levels seen in these two cell types are the result of the same or similar cellular defects, the comparative effects of a number of physical and chemical manipulations on these levels have been compared. The results of these experiments suggest that the elevated levels of free cystine which are characteristic of cystinotic and I-cell fibroblasts may arise from dissimilar cellular abnormalities.

Project Description:

Objectives - Previous work in this laboratory has established that cultured cells derived from individuals affected with the unrelated genetic disorders cystinosis and mucolipidosis II (I-cell disease) exhibit abnormally high intracellular levels of free cystine. Experiments carried out in the preceding year have dealt with the comparative characteristics of cystine accumulation in these two cell types in an effort to ascertain whether a common defect underlies the accumulations of cystine seen in these cells.

Methods Employed - Skin fibroblasts from patients with cystinosis and I-cell disease are grown continuously in culture under a variety of conditions and, after harvesting, are subjected to cystine analysis by a highly sensitive and specific method of assay.

Major Findings -

1. The abnormal accumulations of free cystine in both cystinotic and I-cell fibroblasts have been found to be localized almost exclusively in the lysosomal fractions of these cells.

2. The elevated cystine contents of both types of cultured cells can be markedly reduced by incubation of the cells in media containing low concentrations of the disulfide-reducing agent cysteamine.

3. Incubation of cystinotic cells at somewhat elevated temperatures (e.g. 39-40^{o}C) results in marked diminution of the cystine content in comparison with control cells grown at 35-36^{o}; in contrast, similar incubation of I-cells at the same elevated temperatures is without effect on the cystine content. Conversely, at temperatures lower than normal (e.g. 25^{o}C), the cystine content of cystinotic cells increases somewhat in comparison with cells grown at the normal temperature, whereas similar incubation of I-cells at 25^{o} has little or no effect on intracellular free cystine.

4. Incubation of I-cell fibroblasts in media containing secreted protein from normal fibroblasts results in marked diminution of intracellular cystine; in contrast, there is little or no change in the cystine content of cystinotic fibroblasts when incubated under identical conditions in the presence of the same secretions.

The foregoing results suggest that the abnormally high intralysosomal levels of free cystine in cultured cystinotic and I-cell fibroblasts may arise from dissimilar cellular or metabolic abnormalities.

Significance - Despite much study, the underlying cause of human cystinosis is unknown. It is hoped that the study of the factors which affect the intracellular storage of cystine in cystinotic and I-cell fibroblasts will contribute to an understanding of the pathogenetic mechanism of cystinosis.

Proposed Course of Project - Studies on the effect of various physical and chemical factors on the intracellular free cystine contents of cystinotic and I-cell fibroblasts will continue. In addition, collaborative research with Dr. J. D. Schulman and Dr. R. Steinhertz of NICHHD on the comparative rates of efflux of isotopically labeled cystine from lysosomes of normal and cystinotic leukocytes, initiated during the course of the preceding year, will continue.

Publications:

Tietze, F., Butler, J. D.: Elevated cystine levels in cultured skin fibroblasts from patients with I-cell disease. Pediatric Research 13: 1350-1355, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 18003-07 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Polyamines in the Hormone-dependent Growth and Development of Mammary Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Takami Oka	Research Chemist	LBM	NIAMDD
OTHER:	Yasunori Nagamatsu	Visiting Fellow	LBM	NIAMDD
	Nobuyuki Terada	Visiting Fellow	LBM	NIAMDD
	John Perry	Research Assistant	LBM	NIAMDD
	Masayoshi Ono	Guest Worker	LBM	NIAMDD

COOPERATING UNITS (if any)

None

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5.0	4.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) S-adenosyl-L-methionine decarboxylase, a key enzyme in polyamine biosynthesis, has been purified to apparent homogeneity from mouse mammary gland and liver. By use of the purified enzyme, a monospecific mouse antibody to the enzyme has been prepared. The antibody was used in measuring levels of the enzyme during hormone-dependent development of the mammary gland.
2) Alpha-lactalbumin, a specific milk protein, from mouse milk has been purified to apparent homogeneity, and its antibody has been prepared. The antibody has been used to study the hormonal regulation of milk protein synthesis.
3) By use of immunochemical assay for milk proteins, a striking difference has been found between the optimal concentrations of cortisol required for maximal induction of casein and alpha-lactalbumin: 3×10^{-8} molar for alpha-lactalbumin and 3×10^{-6} molar for casein. Moreover, 10^{-7} to 10^{-5} molar cortisol caused progressive inhibition of alpha-lactalbumin. By the use of different hormone combinations and decreased concentration of cortisol, we have been able to identify cyclic AMP as a negative controlling factor for the induction of milk protein synthesis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives - Elucidation of the fundamental mechanisms involved in the regulation of the growth and development of mammalian cells is one of the important problems in the field of biochemistry and cell biology. Our objective is to assess possible involvement of some intracellular regulatory substances in the developmental processes. We have used an in vitro model system wherein the mammary gland undergoes cell proliferation and functional differentiation under the influence of insulin, glucocorticoid and prolactin.

Methods - The experimental procedures employed in this investigation include the following: organ culture of mouse mammary gland; preparation of mammary epithelium free of fat cells; isolation and characterization of mouse milk proteins, casein and alpha-lactalbumin using a number of column chromatography techniques; development of specific antibodies to the milk proteins and of the immunochemical assay methods; isolation, characterization of mammary milk protein mRNAs and cell-free translation of these mRNAs; characterization of macromolecules by sodium dodecyl sulfate polyacrylamide gel electrophoresis; enzyme assays for ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase, UDP-galactosyl transferase and alpha-lactalbumin.

Major Findings - Synergistic actions of insulin, glucocorticoid and prolactin induce proliferation of the epithelial cells and formation of the milk proteins casein and alpha-lactalbumin in organ culture of mouse mammary gland. During the course of investigation of the mechanisms involved in the hormonal regulation of milk protein synthesis, immunochemical method to determine the synthesis of casein and alpha-lactalbumin as well as a quantitative assay method to measure the amount of alpha-lactalbumin have been developed following the isolation and purification of the two milk proteins. Using these methods, it was found that the synthesis of casein and alpha-lactalbumin are not synchronous and subject to the differential, concentration-dependent action of cortisol: the optimal concentrations of cortisol required for maximal induction of casein and alpha-lactalbumin are 3×10^{-6}M and 3×10^{-8}M, respectively. Moreover, 10^{-7} to 10^{-5}M cortisol caused progressive inhibition of alpha-lactalbumin accumulation. By the use of optimal culture conditions for the induction of the two milk proteins, cyclic AMP has been shown to act as a negative regulator of hormonally induced lactogenesis in mammary cells.

The hormonal stimulation of polyamine biosynthesis in cultured mammary explants is mediated by increases in the activity of various enzymes in the polyamine biosynthetic pathway. The activity of ornithine decarboxylase, which catalyzes the formation of putrescine from ornithine, increases in a biphasic manner. The first peak of activity is caused largely by incubation of tissue explants in a hormone-free medium and is sensitive to the osmolarity of the culture medium. Experiments employing various culture conditions in which the intracellular level of putrescine was varied by alterations in the osmolarity of culture medium, use of several inhibitors of ornithine decarboxylase, and use of mammary explants containing varying amounts of endogenous level of putrescine indicate that an early rise in the level of ornithine

decarboxylase and putrescine is essential for hormonal stimulation of DNA synthesis.

Proposed Course of Project and Significance - We intend to pursue the elucidation of the complex regulatory mechanism involved in the growth and development of the mammary gland. Emphasis will be placed on investigation of possible intracellular regulatory factors including polyamines, cyclic nucleotides and prostaglandins in the actions of insulin, glucocorticoid and prolactin on the proliferation and differentiation of mammary cells at cellular and molecular levels. Such knowledge would certainly be useful for diagnosis and treatment of various metabolic disorders.

Publications:

Hori, C., and Oka, T.: Induction by lithium ion of multiplication of mouse mammary epithelium in culture. Proc. Natl. Acad. Sci. USA 72: 2823-2827, 1979.

Sakai, T., Hori, C., Kano, K., and Oka, T.: Purification and characterization of S-adenosyl-L-methionine decarboxylase from mouse mammary gland and liver. Biochemistry 18: 5541-5547, 1979.

Nagamatsu, Y., and Oka, T.: Mouse α-lactalbumin; purification, characterication and the antibody preparation. Biochem. J. 185: 227-237, 1980.

Perry, J. W., and Oka, T.: Regulation of ornithinine decarboxylase in cultured mouse mammary gland by the osmolarity in the cellular environment. Biochim. Biophys. Acta 629: 24-35, 1980.

Inoue, H., and Oka, T.: The requirement for putrescine in DNA synthesis by virgin mouse mammary gland cultured in hypotonic medium. J. Biol. Chem. 255: 3308-3312, 1980.

Ono, M., and Oka, T.: Dose-dependent differential actions of cortisol on the induction of casein and α-lactalbumin in virgin mouse mammary gland in culture. Cell 19: 473-480, 1980.

Perry, J. W., and Oka, T.: Cyclic AMP as a negative regulator of lactogenesis. Proc. Natl. Acad. Sci. USA 77: 2093-2097, 1980.

Ono, M., and Oka, T.: α-Lactalbumin-casein induction in virgin mouse mammary explants: Dose-dependent differential action of cortisol. Science 207: 1367-1399, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18004-07 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Lesions in Genetic Mucopolysaccharidoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Irwin G. Leder Research Chemist LBM NIAMDD

COOPERATING UNITS (if any)

Ulf Lindahl, Department of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden

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1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A novel sulfatase, specific for the 3-0 sulfate ester of methyl-2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate, has been purified approximately 70-fold from human urine. By means of definitively synthesized ^{35}S-labeled glucosamine derivatives, it has been established that the enzyme a) is specific for the 3-0 sulfate ester and b) requires that the adjacent amino group be sulfated rather than free or acetylated.

A disulfated glucosamine residue corresponding to the synthetic enzyme substrate is not known to exist in any natural substance. Among the glycosaminoglycans, only heparin and heparan sulfate can readily accommodate a 3-0 sulfated sulfaminoglucose moiety. Using the purified 3-0 sulfatase as a structural probe, evidence has been obtained indicating that both 3-0 sulfated and 3,6 disulfated residues are uniquely present in heparin fragments having a high affinity for antithrombin. The presence of 3-0 sulfated glucosamine residues is an important new modification in the accepted structure of heparin; such residues may play a key role in the mechanism of inhibition of blood coagulation by the heparin-antithrombin complex.

Project Description:

Objectives - To purify and elucidate the specificity, tissue distribution and role of a novel glucosamine sulfohydrolase isolated from human urine.

Methods - 1) Conventional procedures are applied to the purification of the enzyme. Appropriately labeled substrates and simplified column procedures are used to study the enzyme and to detect its possible presence in various tissues.

2) The purified enzyme, in combination with nitrous acid and periodate degradative procedures, is used to probe for the presence of 3-0 sulfated glucosamine elements in heparin.

Major Findings -

1) A novel sulfohydrolase, specific for the 3-0 sulfate ester of methyl-2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate, has been purified approximately 70-fold from human urine. By means of definitively synthesized ^{35}S-labeled glucosamine derivatives, it has been established that the enzyme a) is specific for the 3-0 sulfate ester and b) requires that the adjacent amino group be sulfated rather than free or acetylated.

2) A disulfated glucosamine residue corresponding to the synthetic enzyme substrate is not known to exist in any natural substance. Among the glycosaminoglycans, only heparin, and to a lesser extent heparan sulfate, can readily accommodate a 3-0 sulfated sulfaminoglucose moiety. Using the purified 3-0 sulfatase as a structural probe, evidence has been obtained, in collaboration with Dr. Ulf Lindahl, that both 3-0 sulfated and 3,6 disulfated residues are uniquely present in heparin fragments having a high affinity for antithrombin. The presence of 3-0 sulfated glucosamine residues is an important new modification in the accepted structure of heparin; such residues may play a key role in the mechanism of inhibition of blood coagulation by the heparin-antithrombin complex.

Significance - Considerable progress has been made in clarifying the role of heparin in the interaction of antithrombin and thrombin. Important elements of the high affinity antithrombin binding sequence of heparin have been identified. However, these studies failed to discover any specific structure in high affinity heparin which was not present in heparin having little or no affinity for antithrombin. The studies described in this project, by identifying a new structural element in heparin, may make it possible to completely describe the antithrombin binding sequence of heparin and the nature of the resulting interaction. Such knowledge may lead to the preparation of more convenient and effective anticoagulants.

Proposed Course -

1) The purification of the enzyme will be advanced and its properties and tissue distribution studied.

2) The purified enzyme and specifically sulfated substrate analogues will be used to explore the structure of heparin and its interaction with antithrombin.

Publications:

Leder, I. G.: A novel 3-0 sulfatase from human urine acting on methyl-2-deoxy-2-sulfamino-α-D-glucopyranoside 3-sulfate. Biochem. Biophys. Res. Comm. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18005-07 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Differentiation of Lymphoid Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Milton Kern	Research Chemist	LBM NIAMDD
OTHER:	Stephen H. Gregory	Staff Fellow	LBM NIAMDD
	Sastry Gollapudi	Visiting Fellow	LBM NIAMDD

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The lipid A induced secretion of non-immunoglobulin protein by bone marrow cells derived from responder, nonresponder and low responder mouse strains was observed to correlate poorly with the lipid A responsiveness assessment based on the mitogenic reactivity of splenocytes. This new phenotype should prove useful in characterizing the lipid A response in regard to many biological activities that are known to be controlled at a single genetic locus in responder/nonresponder mice.

A simple method has been devised using a single glass surface for purification, identification and autoradiographic analysis of ^{125}I-lipid A binding by individual macrophage cells derived from spleen and peritoneal fluids.

It has been demonstrated that ATP covalently linked to agarose is mitogenic for T cells at a nominal ATP concentration one tenth the concentration required to achieve the same effect with soluble ATP. The findings are compatible with a membrane site for ATP function as a mitogen.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives - The purpose of this project is to study the differentiation of the cells involved in the immune response with regard to cell types, cell interactions and control mechanisms. During this report period the experimental work emphasized was as follows: (1) establishment of the lipid A induced secretion of non-immunoglobulin protein by bone marrow cells as a novel phenotype useful in screening lipid A responsiveness; (2) development of a novel method using a single glass surface for purification, identification and autoradiographic analysis of ^{125}I-lipid A binding by individual macrophage cells derived from spleen and peritoneal fluids; and (3) study of the characteristics of T cells which respond mitogenically to ATP and ATP covalently linked to agarose.

Methods Employed - Secreted products were labeled by incubating cells in medium 199 supplemented with [^{3}H]-leucine and the extracellular fluids were isolated. Secretory products (non-immunoglobulin) were characterized on appropriate Biogel columns. Autoradiographic and immunofluorescence techniques were also employed.

Major Findings - In earlier studies it was shown that the lipid A moiety of lipopolysaccharide induced the enhanced secretion of non-immunoglobulin proteins by bone marrow cells but not by any of the many other types of cells tested. This novel phenotypic expression of lipopolysaccharide responsiveness was assessed for bone marrow cells derived from lipopolysaccharide-responder, -nonresponder and -low-responder mouse strains and found to correlate only partially with the lipopolysaccharide responsiveness assessment based solely on the mitogenic reactivity of splenocytes. These findings indicate that the phenotype based on the lipopolysaccharide enhanced secretion of non-immunoglobulin protein will be useful in characterizing more precisely the lipopolysaccharide response in regard to the many biological activities that are known to be affected by lipid A although controlled at a single genetic locus in responder/nonresponder mice.

A simple method has been devised using a single glass surface for the purification, identification and autoradiographic analysis of lipid A binding by individual macrophage cells derived from spleen and peritoneal fluids. The finding that individual macrophage cells varied in their ability to bind ^{125}I-lipid A is consistent with the growing body of data indicating the heterogeneity of macrophage cells. This general method is also amenable to other kinds of studies in which the interactions between radiolabeled compounds and individual macrophage cells are of specific interest and should be useful, as well, in detecting possible differentiation antigens whose expression may vary with the maturity of individual macrophage cells within a given population.

In continuation of a study of the mitogenic activity of nucleotides like ATP toward T cells, the extent of ATP stimulation of cell proliferation was examined by simultaneously studying the kinetics of DNA synthesis and kinetics of appearance of radioactivity in individual cells using autoradiography. The

data indicate that proliferation is limited and appears to be restricted to a single round of cell duplication unlike the multiple rounds of cell duplication that have been ordinarily observed with other mitogenic agents such as concanavalin A, lectins and lipopolysaccharide. ATP, rendered insoluble via covalent linkage to agarose, was as effective as soluble ATP when used at one tenth the nominal concentration. It is likely, because of steric considerations involved in bringing agarose particles and cells in contact, that insoluble ATP may be equally as effective as soluble ATP at even lower concentrations (possibly 2-3 orders of magnitude lower). Moreover, these findings are most compatible with a membrane site for ATP function as a mitogen. Preliminary studies of compounds of ATP linked to agarose via different parts of the ATP molecule indicate that linkage via the adenine moiety leads to essentially inactive compounds while linkage through ribose yielded a mitogenic compound. Finally, by several tests it has been observed that ATP and GTP react with different cells in T cell populations although both have about the same mitogenic activity.

Significance to Biomedical Research and the Program of the Institute - A knowledge of the control mechanisms involved in the induction of immunoglobulin production will have vital importance not only in understanding how the organism reacts to foreign antigens, but also may be expected to affect significantly the approach used in regard to a number of immune diseases, e.g., immunodeficiency diseases, allergic disease, autoimmune disease and neoplastic disease of lymphoid cells. An understanding of the activation of lymphoid cells by mitogenic agents like LPS and concanavalin A has direct relevance in characterization of the immune response.

Proposed Course of Project - Attempts will be made to isolate those cells mitogenically responsive to ATP by absorbing and eluting such cells on ATP-agarose. Subsequent to purification, surface components of intact cells will be radiolabeled, subjected to dissociation by several techniques and a search made for a product (presumably the receptor) capable of binding to ATP-agarose. Studies will also be directed toward determining the reason for and mechanism by which the proliferation of ATP stimulated cells is apparently limited to a single division.

The product secreted by bone marrow cells in response to lipid A will be characterized with respect to its structure and function.

Publications

Gregory, S. H., Zimmerman, D. H., and Kern, M.: The lipid A moiety of lipopolysaccharide is specifically bound to B cell subpopulations of responder and nonresponder animals. J. Immunol. (in press).

Gregory, S. H., Zimmerman, D. H., and Kern, M.: (^{125}I)-Lipid A binding by individual macrophage cells: Cell purification, culture and autoradiographic analysis on a single surface. Anal. Biochem. (in press).

Gollapudi, S. V. S., and Kern, M.: Lipid A induces cells, uniquely present in bone marrow, to secrete proteins other than immunoglobulins. European J. Biochem. (in press).

Gollapudi, S. V. S., and Kern, M.: A novel early effect of concanavalin A on thymocytes. Biochem. Biophys. Res. Comm. 88: 804-809, 1979.

Gregory, S. H., and Kern, M.: The Heterogeneous distribution of 5'-nucleotidase among rabbit lymphocytes. J. Immunol. 123: 1078-1082, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18006-09 LBM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Enzymes of Carbohydrate Metabolism in Invertebrates and Vertebrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marjorie R. Stetten	Research Chemist	LBM	NIAMDD
OTHER:	Paul K. Goldsmith	Research Biologist	LBM	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two major isozymes of hexokinase have been found to occur in the hepatopancreas and muscles of Homarus americanus (lobster). They have been separated from each other by DEAE-cellulose ion exchange chromatography and by polyacrylamide gel electrophoresis and their kinetic properties and substrate specificity studied. One resembles type I and II hexokinases occurring in all vertebrates, having a very low K_m for glucose and mannose and a high K_m for fructose and being subject to product inhibition or control by low concentrations of ADP or glucose-6-phosphate. The other differs significantly, so far as we are aware, from any hexokinase which has previously been described. Phosphorylation of glucose is catalyzed only at very high glucose concentrations and is not inhibited by glucose-6-P. In this it resembles type IV hexokinase of vertebrates but it differs from that enzyme in its great affinity for fructose, mannose and 2-deoxy-glucose.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives - To gain insight into the occurrence and normal functioning of enzymes of carbohydrate metabolism in the hepatopancreas and muscles of marine invertebrates and to compare these with corresponding enzymes of liver and muscles of vertebrates.

Methods - Separation of enzymes from tissue homogenates are carried out by centrifugation, DEAE-cellulose ion exchange chromatography and polyacrylamide gel electrophoresis. Kinetic and substrate specificity studies are done by spectrophotometric measurements of rates of appropriate enzyme catalyzed reactions. Quantitative levels of sugars, sugar phosphates and glycogen are measured by standard methods. Where appropriate, specific isotopically labelled sugars are used to study the turnover of tissue glycogen in vivo.

An improved method of measuring hexokinases in crude tissue preparations has been developed. Leuconostoc mesenteroides glucose-6-P dehydrogenase, with NAD as cofactor, is used in the assay to avoid the variable further reduction of nucleotide caused by the action of contaminating 6-phosphogluconate dehydrogenase when NADP is used with yeast glucose 6-phosphate dehydrogenase.

Major Findings - Two major ATP:hexose 6-phosphotransferases (hexokinases) have been identified in tissues of Homarus americanus (lobster) and separated from each other by DEAE-cellulose ion exchange chromatography and by polyacrylamide gel electrophoresis. The molecular weight of each, determined by gel filtration, is about 50,000. Hexokinase II, named for its column elution order, resembles hexokinase isozymes I and II of vertebrates. K_m values for glucose, mannose and fructose are 0.08, 0.13 and 6.7 mM, respectively. It is strongly inhibited by the reaction products, ADP and glucose-6-P (K_i = 0.8 mM). Hexokinase I appears to be different from any animal hexokinase previously described. It has a high affinity for mannose and fructose and low affinity for glucose. K_m values are 6, 0.07, and 1.2 mM and relative maximum rates 100, 520 and 1,070 for glucose, mannose and fructose, respectively. Hexokinase I is not inhibited by physiological concentrations of ATP nor by glucose-6-P, mannose-6-P or fructose-6-P even at high concentrations. Both enzymes occur in muscle at about 10% of the concentration found in the hepatopancreas.

Significance to Bio-medical Research and the Program of the Institute: Any knowledge of normal metabolism in other species may aid in understanding the functioning of man in health and disease.

Proposed Course of Project

1. A phylogenetic survey of isozymes of hexokinase occurring in a variety of invertebrate species will be carried out, using electrophoretic and column chromatographic methods. Of particular interest is an assessment of the species distribution and function of the isozyme with a high K_m for glucose which has been found in lobster.

2. Isolation and characterization of a glucose dehydrogenase which we have found to occur in appreciable concentrations in Limulus hepatopancreas will be attempted. The existance of an initial non-phosphorylative step in the utilization of sugar in this species is a possibility.

3. A study of the inducibility of the hexokinase isozymes of arthropods will be made at the Marine Biological Laboratory, where we can compare fed and fasted animals under essentially normal conditions.

4. Dr. Paul Goldsmith has developed an enzyme-linked immunoassay for human IgE which he is using, in collaboration with Drs. John Gallin and Anthony Fauci of NIAID, in studying the in vitro production of IgE by T and B lymphocytes of patients with recurrent candida infections. He is developing a similar assay for IgG rheumatoid factor and is collaborating with Dr. Jack Karsh in evaluating its presence in patients with known IgM rheumatoid factor related diseases.

Publications: None

ANNUAL REPORT OF THE LABORATORY OF CHEMISTRY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

INTRODUCTION

On June 20, 1980, the Laboratory of Chemistry, formerly Division of Chemistry of the Hygienic Laboratory, USPHS, completed 75 years of active research.

In order to stay active, responsive and perhaps even "relevant" the program of the laboratory and its collaborative base of operation was further broadened. The constant call or pressure for NIH intramural research to be more visible or accountable tends to remove some of the natural shyness of the true scientist to advertise achievements.

If highlights of this year's program were to be singled out, the original raison d'être for the Section on Medicinal Chemistry and its mission came closer to fulfillment with a technologically feasible and simple total synthesis of morphine and of deoxydihydromorphine. The possible consequences of this "breakthrough" are obvious, even to a layman.

The understanding of the molecular biology of interferon should precede its popularization in the Nation's newspapers. An accurate assay of enzymes involved in its induction is a milestone on the long road of understanding and applying this over-advertised defensive protein of the body.

To probe receptors or ion transport in voltage-sensitive channels special toxins or selective agents have to be discovered or synthesized. One of them, perhydrohistrionicotoxin, has now become synthetically available. In addition, phencyclidine, an anesthetic with CNS side-effects, abused as "angel-dust" has now been discovered as a readily available substitute for histrionicotoxin.

A toxin isolated from blue algae, anatoxin-a has been found to be an ideal agonist for nicotinic acetylcholine receptors, surpassing carbamylcholine and superceding it as a pharmacological, and possibly clinical, tool.

The combining area of an antibody with specificity to a neutral mammalian galactan has been mapped.

SECTION ON BIOCHEMICAL MECHANISMS

Chemistry of Imidazoles

2-Trifluoromethyl-L-histidine and 2-trifluoromethylhistamine are easily obtained from L-histidine and histamine by a three-step synthesis (see earlier reports). These compounds have now been converted into new substrate analogues: 2-carboxy (by alkaline hydrolysis); 2-carbomethoxy (by methanolysis) and 2-cyano (by ammonolysis). The 2-cyano analogue was found to decompose during chromatographic purification and it became necessary to devise an alternative synthesis. The α-amino groups of the 2-trifluoromethyl analogues were protected as their tert-butoxycarbonyl derivatives; these materials were then subjected to ammonolysis and the resulting 2-cyanoimidazoles were readily purified. Finally, the protecting groups were removed by hydrogen chloride in ethyl acetate to provide pure samples of the desired compounds. Thus, a variety of new analogues of histidine and histamine have been made easily accessible for biochemical and pharmacological evaluation. [L. A. Cohen and H. Kimoto]

Trifluoromethylimidazoles show the unique property of losing hydrogen fluoride above pH 9 to form metastable difluorodiazafulvenes. Such intermediates, derived from histidine and histamine, may be able to serve as covalent affinity labels for enzyme and receptor binding sites. Since the loss of hydrogen fluoride is very slow at physiological pH, we have been exploring methods to increase reactivity by introduction of an additional substituent into the imidazole ring. Thus, 2-trifluoromethylhistidine was successfully chlorinated at C-4 and the reactivity of this derivative is being examined. In attempts to nitrate the histidine analogue, a novel reaction was discovered whereby strong acid effects the replacement of the trifluoromethyl group by a fluorine atom. Should this reaction prove to be practical on a large scale, it would provide a new and valuable route to 2-fluoroimidazoles, which compounds are obtainable only by photochemical procedures in modest yield. 4-Trifluoromethylimidazoles lose hydrogen fluoride ca. ten times as rapidly as the 2-isomers, and may therefore be of greater value as biological tools. Unfortunately, 4-trifluoromethylimidazoles cannot be obtained by the same direct methods as for the 2-isomers, and classical syntheses of the unknown 4-trifluoromethylhistidine and histamine would require at least ten steps each. We attempted, therefore, to develop nonclassical, direct routes to such compounds and have succeeded in this effort. When simple imidazoles are irradiated in the presence of trifluoromethyl iodide, the trifluoromethyl group becomes attached directly to the ring, mixtures of the 2- and 4-isomers being obtained in roughly equal amounts. This new synthetic method is now being attempted with histidine and histamine. [L. A. Cohen, in collaboration with H. Kimoto, Nagoya, Japan]

Our new synthetic method for 2-nitroimidazoles, involving nitration of the C-2 carbanion with alkyl nitrates, has been extended to the synthesis of 2-nitrohistidine. 2-Nitroimidazoles have found wide use as antibiotics and antiparasitic agents, and are currently undergoing extensive evaluation as sensitizers in radiation therapy and as cytotoxic agents highly selective for cancer cells. Thus, the new synthetic method will permit the synthesis and evaluation of a wide variety of new 2-nitroimidazoles. We have initiated an

investigation into the molecular mechanism of the cytotoxic effect, which is thought to involve interaction with extracellular sulfhydryl groups. To date, we have demonstrated that the decomposition of 1-methyl-2-nitroimidazole by aqueous base is not initiated by displacement of the nitro group or by attack at C-2, but by conjugate addition to the 4,5-double bond of the ring; subsequently, the ring opens to give N-methylurea and glyoxalate. The nitro compound does not react with simple thiols under physiological conditions and the biological reaction would appear to be more complex than has been postulated. [L. A. Cohen, C. Rav-Acha, S. Kondo, K. L. Kirk]

Our discovery of the antileukemic activity of 2-fluorohistidine, coupled with the demonstration that 2-fluorourocanic acid is a powerful inhibitor of urocanase, have led us to speculate that the antileukemic effect may involve a block of the utilization of histidine as a one-carbon donor for purine biosynthesis or for cell division. We have been involved, therefore, in efforts to synthesize a number of analogues of urocanic acid for pharmacological evaluation. Although certain analogues can be obtained on a microscale by deamination of the corresponding histidine analogues with histidine ammonia-lyase, this method is impractical for large quantities. Synthetic methods, on the other hand, are complicated because of the reactive double bond in the side chain. We have now developed a procedure for reduction of urocanic acid to the primary allylic alcohol, urocanol. Following introduction of the desired 2-substituent, the alcohol moiety will be reoxidized to the carboxylic acid. [L. A. Cohen, O. Olubajo, K. L. Kirk]

The first step in the synthesis of 2-trifluoromethylhistidine involves cleavage of the imidazole ring of histidine with benzoyl chloride and strong alkali (Bamberger cleavage). This procedure does not work with urocanic acid or with any imidazole carrying an electronegative substituent. By elucidating the mechanism of the Bamberger cleavage, we were able to predict that certain other acid chlorides should be effective for the less reactive imidazoles. Indeed, Bamberger cleavage of urocanic acid was achieved by use of carbobenzoxy chloride and the product has been converted into 2-trifluoromethylurocanic acid. The latter compound can now serve as a starting point for the synthesis of other 2-substituted urocanic acids. [L. A. Cohen, M. Channing]

Imidazoles exhibit a variety of anomalous physical and chemical properties which can best be explained by considering the ring to be only slightly aromatic, or nonaromatic, in character. We have sought to evaluate the magnitude of pi-electron transmission across the ring by studying the ability of a nitro group to activate the protons of a distal methyl group toward isotope exchange. While activation between positions 4 and 5 is very strong, that between 2 and 4 or 2 and 5 is weak, supporting the argument that an aromatic ring current, if present at all, is not significant. This work also demonstrated that introduction of the N-methyl group effects bond fixation in the amidine portion of the ring, and that the two nitrogen atoms are no longer equivalent in electron transmission. [L. A. Cohen, C. Rav-Acha]

Biochemistry and Pharmacology of Histidine Analogues

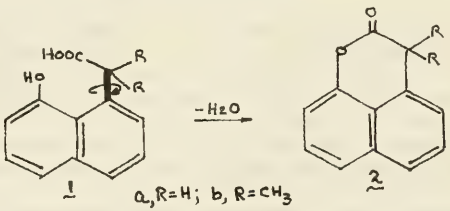
Studies have continued on the incorporation of 2-fluorohistidine into various protein-synthesizing systems, from microorganisms to the whole mouse.

Earlier experiments had involved 2-F-his with tritium labeling in the imidazole ring. The stability of the label to exchange is not quite as high as desirable, and efforts were made to prepare the same compound with a radioactive label on the amino acid side chain. For this purpose, the corresponding malonic acid derivative has been synthesized; decarboxylation in deuterium oxide provides an amino acid with label at the α-position. Hence, the tritiated material will be obtainable by an analogous procedure. [S. Kondo, K. L. Kirk, L. A. Cohen]

Studies on the antibacterial activity of 2-fluorohistidine have shown that the cessation of growth of E. coli is due, at least in part, to a powerful inhibition of the first enzyme in the histidine biosynthetic pathway, phosphoribose-ATP pyrophosphorylase. Further studies have now shown that the inhibition can be overcome by prolonged incubation, and that the resistant strain (presumably a mutant) is no longer sensitive to 2-fluorohistidine. Since we have demonstrated that the analogue is partially incorporated into protein (in place of histidine) during the brief growth cycle, the mutant may be able to incorporate much larger amounts, thus permitting the biosynthesis and isolation of various pseudoenzymes containing fluorine. These analogues are expected to be useful for study of enzyme mechanism by fluorine NMR and by other techniques. [L. A. Cohen, P. A. Cohen]

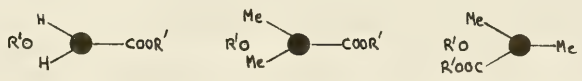
Stereopopulation Control and Models for Enzyme Action

In continuation of our efforts to understand the role of conformational restriction in intramolecular rate enhancement, we have developed synthetic methods for compounds 1a and 1b, and have studied the kinetics of conversion



1 a, R=H; b, R=CH₃ 2

of these compounds to their lactones, 2. The peri-naphthalene system was chosen because of its simplicity, i.e., only one bond (heavy line) can rotate to produce conformational isomers. Compound 1b was found to cyclize at least 10^5 as rapidly as 1a and, even at pH 11, cyclization of 1b is complete within a few seconds. We consider that, in 1a, the preferred rotamer has the carboxyl group positioned as far from the phenol as possible, in order to minimize dipolar repulsion and steric crowding between the bulky solvated dipoles (1a').



1a' 1b' 1b''

In the analogous rotamer for 1b, however, the solvated phenol would have to be flanked by two bulky methyl groups (1b') or by one methyl and a carboxyl (1b"). Since methyl is much larger than carboxyl, the preferred rotamer should now be 1b" and, thus, ring closure would be faster because the latter conformation is much closer to the geometry of the transition state. At ambient temperature, the NMR signal for the methyl groups of 1b (R' = alkyl) appears as a sharp singlet. As the temperature is reduced, the signal broadens and, at -20°, has separated into two distinct singlets of equal area. Since the methyl groups in 1b" are not chemically identical, the low temperature spectrum must represent 1b", supporting the argument based on kinetic data. [L. A. Cohen, M. Channing, M. King]

Chemistry and Biochemistry of Fluorinated Biogenic Amines

The dramatic differences in adrenergic agonist specificities previously described for the isomeric ring-fluorinated norepinephrines (FNE) have been studied in greater detail. To gain insight into the mechanism(s) of these specificities, the binding of FNEs and fluorodopamines (FDA) to adrenergic and dopaminergic receptors in rat brain membranes has been studied in detail. In the NE series, binding to β_1-adrenergic receptors in rat cerebral cortical membranes followed the order: NE > 2FNE > 5FNE >> 6FNE. For β_2-adrenergic receptors, the order was: NE $\sim$ 2FNE $\sim$ 5FNE >> 6FNE. In contrast, for the α-adrenergic receptor, the order of potency was: 6FNE $\sim$ NE > 5FNE >> 2FNE. Thus, the binding studies are in agreement with the previously observed selectivities for the FNEs, i.e., 6FNE is a relatively selective α-adrenergic agonist while 2FNE is a relatively specific β-adrenergic agonist.

2,5,and 6-FDA are more potent ligands for the α_1-adrenergic receptors than for α_2 and β-receptors, but the specificity is independent of fluorine position with respect to the dopaminergic receptor. DA and the three FDAs are essentially equipotent. The lack of major differences in binding of the isomeric FDAs to adrenergic receptors suggests that the specificities shown by FNEs is due to interaction of the ethanolamine side chain with an ortho situated fluorine substituent, and not due to altered phenolic pKa.

Studies on the cAMP generating system in rat cerebral cortex slices, using 6FNE as a specific α-adrenergic agonist, have led to a clearer understanding of the role of the α-adrenergic receptor in cAMP release. Thus, it has been demonstrated that the α-receptor does not stimulate cyclase, but instead potentiates the response of cyclase to endogenous adenosine. [K. L. Kirk, D. Cantacuzene, J. Daly, C. R. Creveling, Y. Nimitkitpaisan, W. Padgett]

Additional analogs of adrenergic agonists have been synthesized in order to try to define more clearly the "fluorine effect" on agonist specificity. Unlike NE, N-isopropylnorepinephrine (isoproterenol) is a relatively pure β-adrenergic agonist. 2- and 6-Fluoroisoproterenol have been prepared and investigated in Guinea Pig heart preparations. Initial results indicate the α-fluoro isomer is a somewhat better β-agonist than isoproterenol while the 6-fluoro derivative shows both α and β-adrenergic agonist activity, thus providing another example of an α-shift mediated by a fluorine substituent in the

6-position. To extend these studies, analogs of phenylephrine (a relatively pure α-adrenergic agonist) have been synthesized having fluorine in the 2- or 6-positions. Results of testing of these analogs will have added significance because of the presence of only one phenolic group on the aromatic ring. To determine the effects of other substituents on NE, 2, and 6-chloro NE have been prepared. [K. L. Kirk, D. Cantacuzene, O. Olubajo, C. R. Creveling, D. McCulloh]

6-Fluoro-α-methyl DOPA has been synthesized and has been shown to be effective (comparable to α-methyl DOPA) in lowering the blood pressure of spontaneously hypertensive rats. [K. L. Kirk, D. Cantacuzene, E. McNeil, C. R. Creveling]

^{19}F NMR Spectra as Probes of Molecular Motion

^{19}F NMR studies of fluorinated molecules in intact platelets have continued. It has been determined that human and pig platelets differ in the mechanism of amine storage (magnesium versus calcium associated, respectively). In pig vesicles, both ATP and serotonin (as judged from the behavior of difluoroserotonin) appear to exist in a gel-like state, whose molecular tumbling rates increase linearly (by about two orders of magnitude) as the temperature is raised from 4°C to 37°C. In human platelets, adenine nucleotides and pyrophosphate appear equally restricted in motion at 4°C and 37°C. Loss of difluoroserotonin from the cell at temperatures above 22°C precludes determination of restricted motion of this molecule at higher temperatures. [K. L. Kirk, J. L. Costa, C. M. Dobson, F. M. Poulsen]

SECTION ON CARBOHYDRATES

Model of IgA J539

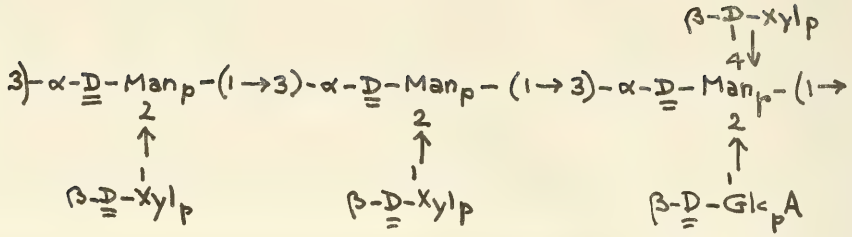
A space filling model of J-539 has been built from the framework of McPC 603. The combining area of the Ig has been lcoated using the data accumulated in this Section over the past number of years. A fitting has been made with galactotriose. It appears that the nonreducing galactose moiety binds near the Tryptophan 33H and the exposed histidine of the CDR-2 of the H-chain. Titrations have shown that it is unlikely that the CDR-3 of the H-chain is involved in binding. The ligand appears to bind in a groove generally extended from CDR-2H towards CDR-1L and CDR-3L.[Drs. Feldmann and Potter]

The Binding of J539 to Linear Galactan

Iga J539 binds intercatenary to linear galactan and precipitates with it. This confirms the suspected groove-site of J539. [Dr. A. Roy]

The structure of the capsular antigen from Cryptococcus bacillisporus serotype B

The antigen was isolated, purified, and its structure determined as:



This completes the structural work on the Cryptococci polysaccharides (A, B, C, D) and opens the way for a close look at the fine-determination for the immune response. [Dr. A. Bhattacharjee]

The immune response to pneumococcal antigens

The response (rabbit) to pneumococcal polysaccharide S-27 is polyclonal (as expected). The antigen was selectively degraded into its repeating unit: a tetrasaccharide with phosphorylcholine (PC) attached.

The antibody pool shows homogeneous binding affinity for this PC-tetrasaccharide but the antibody pool shows heterogeneous binding to various aspects of this tetrasaccharide rep. unit. What this means in that the lymphocytes which have a certain threshold affinity for the entire antigenic determinant will partake in the immune response, and that this threshold value may be due to the affinity of any part of the determinant for any particular Ig on sub-populations in the lymphosite pool. [Dr. L. Bennett]

Solid Phase Synthesis of Linear β1,6-D-galactan

We have prepared all the intermediates for the continuous synthesis of a β1,6-D-galactan and have synthesized the trisaccharide. The intended polysaccharide is of great interest in our work on monoclonal antibodies. [Dr. A. Bhattacharjee]

SECTION ON MEDICINAL CHEMISTRY

Our program to further elucidate the essential structural and configurational features in a variety of important natural products made further progress. Many of our findings required the synthesis of novel compounds by novel methodology.

RETINOIDS AS POTENTIAL CHEMOPREVENTIVE ANTITUMOR AGENTS:

Retinylidene diketones, the condensation products of retinal with 1,3-diketones, are cleaved with aqueous methylamine into retinal and the corresponding 1,3-diketone. The rate of this cleavage is parallel to the observed in vivo activity, suggesting that this type of compound probably functions

as a prodrug of retinoic acid. Oxygenated retinylidene dimedone derivatives were prepared, but found to be less active than the corresponding deoxygenated precursors. (N. Acton, A. Brossi).

MOLECULAR FEATURES OF COLCHICINE ASSOCIATED WITH ANTIMYTOTIC ACTIVITY:

Chemical manipulation of the A-ring and of the B-ring in colchicine has led to the understanding that substitution at C-3 and C-7 is critical for antitumor activity. The nature of substitution required for optimal activity has been studied and attempts are now being made to make simultaneous changes at both positions. Colchifoline has been detected as a minor alkaloid in commercial samples of colchicine and synthesized by novel methodology. The structure of "epoxycolchicine" has been revised. It was found that all compounds active in vivo did bind to tubulin in vitro but no clear relationship seems to exist between the two tests. Toxicity in mice was determined with all compounds and introduced as a third major criterion in our attempts to find a better colchicine. (H. G. Capraro, L. Atwell, M. Rösner, M. A. Iorio, A. E. Jacobson, and A. Brossi).

MODIFICATION OF THE ANTIMALARIAL MEFLOQUINE:

The synthetic antimalarial mefloquine has been converted into an aziridine intermediate. The latter has photochromic properties and has proved to be a useful intermediate for structural modification within the mefloquine molecule. Insight into the structure-activity requirements of this interesting amino alcohol antimalarial are planned. (M. Rösner and A. Brossi).

(±)-PERHYDROHISTRIONICOTOXIN:

The published syntheses of this interesting frog toxin are cumbersome and difficult to repeat. Further improvements still have to be made if synthetic material can be substituted for that so far obtained from natural sources. Investigations with intermediates obtained by the Kishi route have revealed that the amyl side chain is not required for biological activity and that both, the nitrogen and oxygen functionalities can be changed, without loss of biological activity. (C. P. Mak, K. Takahashi and A. Brossi).

NOVEL SYNTHESES OF MORPHINE AND 3-DEOXYDIHYDROMORPHINE:

Racemic dihydrothebainone and rac. dihydrocodeinone, already converted into morphine and codeine by others, were synthesized by Rice from 3-methoxyphenethylamine. The synthesis is straightforward, simple, and amenable to scale up. Optical resolution of an intermediate tetrahydroisoquinoline has been accomplished. The Rice synthesis will be evaluated on a larger scale for the preparation of congeners of (+)-morphine, compounds otherwise inaccessible. A first total synthesis of (±)-3-deoxydihydromorphine, prepared earlier in optically active form from morphine, has succeeded. Improvements have been made and the synthesis will now be tailored to elaborate this interesting class of compounds further. Intermediates in our synthetic programs were tested as analgesics and in various CNS-screens. It could be shown that the nature and position of the aromatic oxygen functionality is important to ascertain optimal biological effects. This investigation will now be extended to the

dihydromorphine series and may ultimately help to design improved analgesic compounds. The introduction of an aromatic hydroxy group in 3-deoxydihydromorphine, formally yet another synthesis of morphine, is being conceived with chemical and biochemical methods including microbial fermentation and enzymic reactions. Racemization of optically active norreticuline, an important intermediate in the plant biosynthesis of morphine, has been achieved. Either of the two enantiomers can now be racemized, affording a better overall yield of the desired optical isomer. (K.C. Rice, F. Hsu, D. Rozwadowska, L. Atwell, K. C. Choe, A. E. Jacobson, A. Brossi).

TETRAHYDROPAPAVEROLINE (THP):

THP, its optical isomers, and related compounds were studied in their binding to various receptors from rat cerebral cortex. Various O-methyl ethers of THP as well as brominated analogs were synthesized. One of these derivatives, 6'-Br-THP, is highly active in the dopaminergic receptor assay and α-adrenergic assay. (A. E. Jacobson, K. C. Rice, C. P. Mak, J. Reden, A. Brossi).

IRREVERSIBLE INHIBITORS OF THE BENZODIAZEPINE RECEPTORS:

Several novel benzodiazepines containing reactive functional groups were synthesized as specific and irreversible inhibitors of the benzodiazepine receptors. It is hoped that information will be gained which will be of value in further study and characterization these receptors. (K. C. Rice).

SECTION ON METABOLITES

The thrust of this Section is directed to three diverse fields: to the antiviral activity of "fraudulent nucleosides", to the induction and molecular biology of interferon and to an expanding collaborative program on selective manipulation and inhibition of ionic transport both in the ion transport system of the nicotinic and muscarinic acethylcholine receptors as well as in the voltage-sensitive ionic channels for Na^+, K^+, and Ca^{++} in electrogenic membranes of nerve and muscle.

2',5'-Oligo A Synthetase in Human Lymphobastoid Cell Lines (Johnston, Zoon, Friedman and Torrence).

Namalwa cells originate from Burkett's lymphoma and thus have been transformed by Epstein-Barr Virus, although they contain only a few incomplete copies of the virus in their genome and do not shed the virus. We have found that Namalwa cells have levels of 2',5'-oligo-A synthetase activity that equals or exceeds the levels associated with interferon-treated mouse L cells. Newcastle-Disease Virus infection of Namalwa cells enhance the level of synthetase by 2-4-fold while interferon is produced. Treatment of Namalwa cells with exogeneous interferon also slightly enhances synthetase activity levels. Namalwa cells are constitutive producers of interferon (5-15 units/ml), so that the possibility exists that they may activate themselves by constitutive production of interferon. When Namalwa cells are grown for three to four generations in the presence of excess antibody to F and Le interferon,

synthetase activity drops three to ten-fold compared to untreated cells. This decrease in activity can be reversed by NDV infection to produce sufficient interferon to overwhelm the antisera absorption capacity. The synthetase activity that persists in the presence of excess antibody is still quite substantial and remains in the range of levels associated with some interferon-treated cells. It is established that interferon must be externalized to induce an antiviral state. Must it also be externalized to induce 2',5'-oligo A synthetase? Anti-interferon serum decreases synthetase activity levels to those found in 1788 cells, a lymphoblastoid line which does not produce interferon constitutively. Since these 1788 cells have levels comparable to an interferon-treated mouse L cell, we are investigating whether or not lymphocytes themselves may have such elevated 2',5'-oligo A synthetase levels insofar as EB virus transformation of B lymphocytes gives rise to the above lymphoblastoid lines (Johnston, Torrence, Friedman and Berger).

Chemical Synthesis of Adenylyl(2'→5')Adenylyl(2'→5')Adenosine (Imai and Torrence).

A complete and unequivocal chemical synthesis of the "core" of 2',5'-oligo A has been completed in an overall yield (from 5 and 1) of > 40%.

This synthetic material showed the same proton and phosphorus nmr as "core" prepared in cell-free extracts of interferon-treated cells. It also comigrates with radiolabeled biosynthetic "core" on HPLC and a DEAE-cellulose column (linear volt gradient).

A similar synthesis has been reported by Olgivie and Theriault [Tetrahedron Lett. 2111 (1979)].

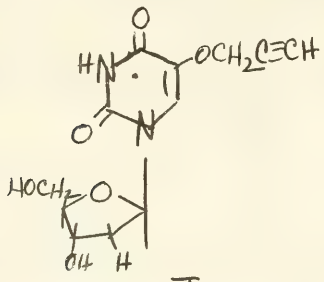
This synthesis not only verified the prepared structure of 2',5'-oligo A, but provides a feasible synthetic route to analogs of 2',5'-oligo A.

Structural Requirements of Nucleic Acids for Activation of 2',5'-Oligoadenylate Synthetase (P. Torrence, M. I. Johnston, R. M. Friedman).

Using an assay (previously developed in this lab) that involves adsorption of 2',5'-oligo A synthetase to 2',5'-ADP-sepharose, we have examined the polynucleotide structural requirements for activation of 2',5'-oligo A synthetase. All single-stranded, triple-stranded and double-helical polymers substituted at the ribose C2' position were unable to activate the enzyme. Other polymers varied over 1000-fold in their ability to activate the enzyme. Poly(I)·poly(s^2C), poly(A).poly(U) and duplexes based on poly(c^7A) were weak activators. In order of decreasing potencies, the strongest activators were P. chromogenum dsRNA ≈ poly(I)·poly(C) > poly(c^7I)·poly(C) > poly(I)·poly(br^5C) ≈ poly(c^7I)·poly(br^5C) > bactiophage QB RNA. These data suggest that the enzyme is quite sensitive to small changes in number and structure.

Synthesis and Anti-Herpes Activity of 5-Cyano-methyleneoxy-2'-deoxyuridine (G.-F. Huang, Okada, E. DeClercq, P. Torrence).

To study the influence of substitution of CN for C≡CH in the anti-herpes virus nucleoside 5-propynyloxy-2'-deoxyuridine (I), 5-cyanomethyleneoxy-2'-deoxyuridine (II) was prepared. When the potassium salt of 5-hydroxy-2'-deoxyuridine was reacted with iodoacetonitrile in dry DMF, the bisalkylated product, 3-cyanomethyl-5'-cyanomethyleneoxy-2'-deoxyuridine (III) was the major product with a lesser amount of 3-cyanomethyl-2'-deoxyuridine (IV) and only a trace of the desired product (II). By constrast, when 5-hydroxy-2'-deoxyuridine was alkylated in water in the presence of one equivalent of KOH, compound II was the major product. Compound II showed an antiherpes virus activity comparable to I and araA in cultures of primary rabbit kidney (PRK) cells. Compound II did not inhibit incorporation of [Me-^{3}H]-dThd or [1',2'-^{3}H]dUrd into DNA of PRK cells; however, its anti-herpes virus activity was completely reversed by either dThd or dUrd. These results suggest that substantial variation of the lipophilicity of the R group of the OCH_2R side chain at pyrimidine C-5 has little effect on the anti-herpes activity of such thymidine analogs.



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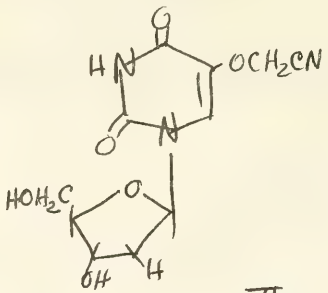

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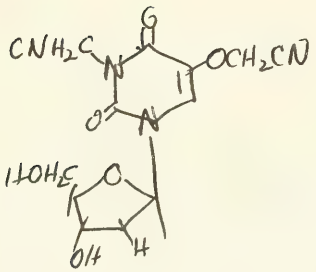

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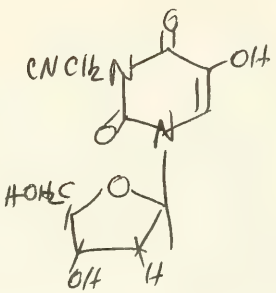


IV

Survey and Outlook (P. Torrence)

The antiviral and antitumor agent interferon appears to induce the synthesis of at least three new enzymatic activities. The first of these uses ATP as a substrate and when "turned on" by double-stranded RNA, synthesizes the novel 2',5'-linked oligonucleotide called 2',5'-oligo(A). This oligo(A) is unique in that it is the first naturally occurring substance with 2',5' rather than 3',5'-phosphodiester bonds. 2',5'-Oligo(A) is an extremely potent protein synthesis inhibitor. A second induced enzyme is a phosphodiesterase which degrades 2',5'-oligo(A) and also apparently cleaves the CCA terminus of transfer RNA. Finally, a third activity, a protein kinase specifically activated by double-stranded RNA, phosphorylates a protein P_1 of 67000 Daltons molecular weight. Recent evidence suggests that some or all of these enzymes may be involved in the establishment of the antiviral state in response to interferon treatment. It is also possible that one or more of these activities might be involved in the mechanism of antitumor action of interferon and perhaps the control of development and differentiation in normal cells.

Our research is currently directed along the following paths:

1. The determination of the relationship of 2',5'-oligo(A) structure to capacity of inhibiting protein synthesis. This will be achieved by the synthesis of various base- and sugar-modified analogs of 2',5'-oligo(A). This study may enable us to find inhibitors of the action of 2',5'-oligo(A), extend the biological half-life and cell-penetration ability of 2',5'-oligo(A) and eventually to attempt to explore the 2',5'-oligo(A) system in a chemotherapeutic sense.

2. Development of a radioimmunoassay for 2',5'-aligo(A). Of the two methods currently available for assay of 2',5'-oligo(A), one is of limited sensitivity, and the other is extremely time-consuming. Thus a much faster, sensitive assay will permit to obtain information on the level of 2',5'-oligo(A) in intact cells under various conditions.

3. Development of a speedy and reliable assay for the 2',5'-oligo(A) phosphodiesterase. A convenient and reliable assay for this enzyme has not yet been described. Since this enzyme represents a potential mechanism for control of 2',5'-oligo(A) levels in cells, a reliable method for its estimation would be invaluable.

4. Nucleic acid structural requirements for activation of the 2',5'-oligo(A) synthetase and the 67K Dalton protein kinase are being investigated in order to determine if there might be any relationship between these enzymes and interferon induction.

5. The possible biological roles of 2',5'-oligo(A) synthetase and 2',5'-oligo(A) itself are being investigated in human leukocytes and lymphoblastoid cell lines.

Phencyclidine, a Convenient Substitute for Histrionicotoxins (E. X. Albuquerque, M.-C. Tsai, R. S. Aronstam, B. Witkop, A. Eldefrawi, and M. E. Eldefrawi).

So far, the histrionicotoxins have fulfilled an important function in blocking the transport of ions, especially of potassium, in the transport system of the postsynaptic membrane containing the acetylcholine receptor. The easily accessible phencyclidine, 1-(1-phenylcyclohexyl)piperidine, "angel dust" has now been studied in detail. The effects of phencyclidine (PCP) were studied on the electrogenic and chemosensitive properties of the neuromuscular junction of skeletal muscle as well as on the binding sites on the acetylcholine (AcCho) receptor and its ionic channel in the electric organ membranes of the electric ray. The directly elicited muscle twitch was markedly potentiated by prolonging the falling phase of the muscle action potential and blocking delayed rectification. The indirectly elicited muscle twitch was transiently potentiated and then blocked by PCP at concentrations below 60 μM. PCP blocked miniature endplate potentials and AcCho sensitivities at the junctional region of innervated muscle, blocked the extrajunctional sensitivity of the chronically denervated muscle, and significantly depressed the peak amplitude of the endplate current (EPC) in a voltage- and time-dependent manner. PCP also caused acceleration of the time course of EPC decay and shortening of the mean life-time of the open ionic channel. The effects of PCP were not due to inhibition of AcCho receptor sites because PCP did not protect against the quasi-irreversible inhibition of receptor sites by α-bungarotoxin, nor did it inhibit binding of [^{3}H]AcCho or ^{125}I-labeled α-bungarotoxin to the receptor sites. On the other hand, PCP blocked the binding of [^{3}H]perhydrohistrionicotoxin to the sites of the ionic channel of the AcCho receptor. The data suggest that PCP reacts with the electrogenic K^{+} channel and the ionic channel associated with the AcCho receptor in the open as well as the closed conformation.

Anatoxin-a, an Ideal Agonist for the Nicotinic Acetylcholine Receptor (E. X. Albuquerque, C. Spivak and B. Witkop).

The naturally occurring toxin of Anabaena flos-aquae, a cyanophyte, had been known to possess strong polarizing activity, but its mode of action as the strongest agonist known for the cholinergic system was brought to light in a collaborative study with the Department of Pharmacology and Experimental Therapeutics by the following findings:

Anatoxin-a, a semirigid, bicyclic amine, caused a depolarizing blockade of the indirectly elicited twitch in frog sartorius muscle. Concentration-response studies of contracture in the rectus abdominus and depolarization in the sartorius muscles of the frog showed that it is the most potent of the nicotinic agonists. It produced desensitization, and the kinetic and steady-state parameters found from chronically denervated rat soleus muscle were similar for anatoxin-a and acetylcholine. In voltage clamped frog sartorius fibers, anatoxin-a induced endplate currents and concommitant increases in endplate current noise. Fourier analysis of this noise revealed that the average single channel lifetime was indistinguishable from that induced by acetylcholine; the single channel

conductance was about 25% less for the toxin compared to acetylcholine. Analysis of nerve evoked endplate currents of spontaneous, miniature endplate currents indicated that the toxin did not alter the time constants for decay or the linearity of the current-voltage relationship. We conclude from this evidence that anatoxin-a does not block the open ion channel and does not produce a voltage-dependent blockade of the closed ion channel. The activity of this unusual agonist may be related to the coulombic and hydrogen bond free energies of binding to the receptor.

The Toxin of the Ladybug, Adaline (E. Gössinger and B. Witkop).

The toxin of the ladybug, in analogy to anatoxin-a, is a basic ketone derived from granatanone, a homolog of tropanone. Its structure is 1-pentyl-9-azabicyclo[3.3.1]nonan-3-one. It has no effect on nicotinic receptors. Its hydrogenation product with a secondary alcoholic group in the pseudotropine conformation, is a competitor for acetylcholine in muscarinic receptors. Synthetic studies in this series have now opened an easy road to the two compounds by intramolecular 1,3-dipolar addition of suitably substituted piperidine nitrones.

Natural Agonists with Muscarinic and Nicotinic Activity.

The clinical administration of arecoline to humans in programs to study learning and sleep patterns has reawakened interest in the finding that quaternization of a muscarinic agonist, such as arecoline, produces a nicotinic agonist or inhibitor. This kind of phenomenon is now studied with pilocarpine.

Alkanol Esters as Analgesics, Anti-epileptics, Anesthetics, CNS Agents and Selective Blockers of Ion Transport in Receptors and Electrogenic Membranes (J. A. Waters).

Aromatic, heterocyclic and cycloalkyl carboxylates of the 4-piperidinol system and related tertiary aminoalcohols have been synthesized for pharmacological and biochemical study. Two CNS classes have been found: (1) non-addicting analgesics (mice, rhesus monkeys); (2) antiepileptics (mice). A tritiated piperidinol ester has been prepared for non-opiate receptor study. Certain compounds have been selected for the study of their effect on the Na^+/K^+ channels of electrogenic membranes. Quantitative and qualitative structure-activity relationships have been made.

Sixteen esters of 1-methyl-4-piperidinol, from a group of one hundred and twenty-six synthetic compounds, exhibited analgesic activity in the codeine-morphine range (hot-plate). In general, these non-quaternary C-4 piperidinol esters showed no physical dependence of the morphine-type. Self-injection behavioral studies indicated very low or no abuse potential (monkeys). Sixty-six compounds were assayed for antiepileptic activity (MES). Those substituted at the 2 position of the aryl ring with phenyl, benzyl or phenethyl exhibited antiepileptic activity in the phenobarbital-methsuximide potency range. This is a relatively strict structural requirement for enhancing antiepileptic activity in these piperidinol esters.

New analgesics which lack physical dependence, abuse liability and other side-effects of the morphine-type narcotics are needed for clinical use and for use in addiction programs. The recent finding of long-term toxic effects of some of the standard antiepileptics (i.e., Dilantin) increases the need for drugs with greater CNS selectivity. The study of these substrates at specific receptor sites and their effect on ion transport will provide a better understanding of their biochemical mechanisms.

A tritiated substrate in the analgesic series will be used to study agonist-agonist interactions in an effort to identify a non-opiate type receptor. Studies will also be made on selected piperidinol esters to determine their effect on the Na^+/K^+ channels of electrogenic membranes. NINCDS is examining several compounds in their advanced antiepileptic assays.

Synthetic Approaches to Frog Toxins Derived from Indolizidine (T. F. Spande).

Alkoxycyclopropanes have been utilized to find a novel approach to substituted indolizidines. This synthetic route is being developed to make accessible indolizidine alkaloids coccurring in frogs, such as Dendrobates histrionicus.

SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

Service Functions and Instrumentation

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses include: GC/MS spectrometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrometry, amino acid analysis, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request. (D. F. Johnson, H. J. C. Yeh, W. R. Landis, P. Parisius, B. Baer, A. Wong, N. Whittaker, B. Torain).

Characterization of the Dehydrogenase-Isomerase Enzymes and Metabolic Pathways in Tetrahymena Pyriformis

The protozoan Tetrahymena pyriformis has steroid dehydrogenases that are similar in functional properties to the dehydrogenases of higher eukaryotes. Specifically, our prior identification of 4-androstene-3β, 17β-diol and

pregnenolone as transformation products from the incubation of T. pyriformis with testosterone and progesterone, respectively, indicated that this protozoan possesses a dehydrogenase-isomerase system that can isomerize a Δ^4-steroid to a Δ^5-steroid and reduce the C-3 carbonyl to a 3β-hydroxyl. The characterization of the dehydrogenase is being pursued.

The secondary alcohol dehydrogenase (SADH) is obtained as a crude supernatant from a 100,000 x g centrifugation of ruptured cells. Purification steps are DEAE-cellulose, ammonium sulfate precipitation, affinity chromatography on Cibacron Blue F3G-A Sepharose, and chromatography on Sephadex G-100. The amount of enzyme activity is low, requiring multiple preparations of the early purification steps to provide sufficient activity to measure in the last step.

The dehydrogenase activity with steroidal and non-steroidal substrates co-purifies. Other evidence supporting the hypothesis that both activities reside in the same protein include: similar heat denaturation behavior; similar behavior with potential inhibitors; and the same pH optimum.

Kinetic studies are limited by the low solubility of the substrates, but the apparent K_m and v_{max} are being determined for a number of steroidal and aliphatic alcohols.

Characterization of the SADH will include pH optimum, molecular weight, isoelectric point and heat inactivation.

Cultures of T. pyriformis will be grown in the presence of varied concentrations of unlabeled steroids. Growth inhibition will be measured by cell counting in a hemocytometer. A fixative technique has been developed to immobilize the protozoa and Trypan blue will be used to distinguish between viable and nonviable intact cells. (N. S. Lamontagne and D. F. Johnson).

Applications of NMR in Biochemical and Biological Systems

Basic information on the structure and molecular interactions of biologically important materials is of significance to biochemical and biological research. One of the most promising approaches in obtaining such information is through the use of nuclear magnetic resonance. The purpose of this project is to develop and apply nuclear magnetic resonance methods for studying molecular structures and molecular interactions in biochemical and biological systems.

Hemerythrin, a non-heme oxygen-carrying protein found in the erythrocytes of certain marine invertebrates, normally occur in an octameric form of molecular weight of 108,000 daltons, and can be dissociated into the monomeric species in the presence of several organic mercurials. Each subunit contains two atoms of iron and can reversibly bind one molecule of oxygen. Oxidation of either oxyhemerythrin or deoxyhemerythrin gives the iron (III) species, methemerythrin, which coordinates a number of simple anionic ligands to give a series of characteristic methemerythrin complexes.

In order to gain information on the conformation and the nature of intersubunit interaction of the protein, we have used NMR spectroscopy to examine the titration behaviors of histidine ring C-2 proton resonances of P. goudii hemerythrin and its subunit A and B, and myohemerythrin from T. zostericola with a number of ligands. Spectral comparison of the monomeric hemerythrins obtained from these species has enabled us to assign several histidine resonances and to characterize their local structures. These include two histidine resonances in hemerythrin A (His-82 and His-34) and one each in hemerythrin B (His-34) and myohemerythrin (His-57). The readily titrable His-82 (pK 6.5) in hemerythrin A and His-57 (pK 6.4) in myohemerythrin in the pH range 4 to 9.5 suggest that these histidines probably are exposed near the surface of the protein. That His-34 in hemerythrin A and hemerythrin B can be observed only below pH 4.5 indicates that His-34 may be partially buried inside the protein or hydrogen-bonded to nearby amine acid residues. The results eliminate the possibility of His-32, His-34 and His-57 as ligands to the iron atoms and hence imply that a maximum number of five histidines are coordinated to the iron atoms.

We have examined the histidine C-2 proton titration behaviors of both octameric and monomeric methemerythrins with several anions (e.g. N_3^-, SCN^-, Cl^-, CN^-, and F^-). The titration data for His-34 were found noticeably different between ligands such as N_3^- (or SCN^-) and Cl^- (CN^- or F^-), and pKa values of His-34 were found considerably higher for the monomeric than the octameric methemerythrins. These findings tend to provide evidence of conformational differences among these hemerythrin complexes and the involvement of His-34 in the intersubunit interactions.

Neurotoxins from snake venoms are a group of small proteins which block the neuromuscular transmission at the post-synaptic membrane by high affinity binding to acetylcholine receptors and accordingly are a subject of considerable interest in the field of neurochemistry. The toxins show a high degree of sequence homology and 13 amino acid residues are found to be invariant among more than 50 neurotoxins being investigated. Considerable attention has been devoted to the investigation of the roles played by some of the amino acid residues in the binding to the receptor and in the lethal activity of the toxins. Basic information on the conformation and structure of the toxins in solution is of importance to the understanding of the nature of toxin-receptor interaction. Although the main chain conformation of these proteins has been elucidated in some detail, relatively few investigations on the local structures of the amino acid residues of the toxins have been reported. In order to gain understanding of the structure-toxicity relationship for these toxins we have undertaken an exploration of the local structure of the toxins using nuclear magnetic resonance as a tool in assessing the detailed structural information of these toxins.

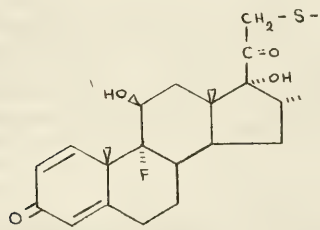
The results of our preliminary ^1H and ^{13}C NMR work on cobrotoxin and pelamitoxin have revealed some detailed structural information, including the micro- environments of His-4, Tyr-25 and His-32 of cobrotoxin and His-30 and His-54 of pelamitoxin. The microenvironments of the two histidines in both cobrotoxin and pelamitoxin are found different as reflected from the differences in the chemical shifts of the C-2 ring protons and pK_a values of these histidines. In combination with deuterium and tritium exchange experi-

ments, which utlilize the difference in isotope exchange rates of the imidazole C-2 hydrogens between the two histidines, the two titratable resonances at δ=7.7 and 7.8 ppm at pH=9.0 for cobrotoxin are assigned to the C-2 ring protons of His-4 (pK_a=5.1) and His-32 (pK_a=5.7), respectively. The readily titratable property of both histidines indicates that they are accessible to solvent water molecules. That the pK_a value of His-4 is lower by 0.6 unit than that of His-32 suggests the presence of a positively charged amino group(s) near His-4. A significant difference in the conformation between the two histidines is also evidenced from the pH-dependence of the ^{13}C chemical shifts of their imidazole carbons. His-4 is found to exist predominently as a 1,5 tautomer, while His-32 as 1,4 tautomer. A detailed structural study of these toxins, including some chemical modification, is now in progress. (H. J. C. Yeh, D. F. Johnson, S. Chiang, C. L. Chatterjee).

Nature of Steroid Receptor Interactions

Steroid specific events are induced by nanomolar concentrations of hormone in responsive cells. Thus, extremely sensitive methods are required in order to monitor the steroid at physiological levels. Fluorescence spectroscopy can 1) detect some compounds at concentrations as low as 10^{-12} M and 2) provide information about the microenvironment of the fluorescent molecule. These two properties make fluorescence spectroscopy ideally suited for a study of steroid-receptor interactions. Due to the tendancy of receptor-steroid complexes to dissociate, a covalently bound fluorescent receptor-steroid complex would be even more useful. In order to obtain such a complex, we have recently developed a new methodology for affinity labelling which we call fluorescent chemo-affinity labelling (FCAL). In FCAL o-phthaladehyde specifically cross-links primary amines and thiols to give strongly fluorescent 1-alkylthio-2-alkylisoindoles in high yields. In collaboration with Herman Ammon of Univ. of Md., we are trying to obtain an x-ray structure of an isoindole derivative in order to unambigously prove the isoindole structure and to answer some basic theoretical questions about this ring system.

In order to obtain a fluorescent, covalent receptor-steroid complex, we have prepared two series of hydrolytically stable glucocorticoid derivatives (1 and 2) which should participate in the FCAL reaction. In model studies with



1) X = SH (n = 2-6)

2) X = NH_2 (n = 2-6)

OPTA and an added amine or thiol, each steroid gives a good yield of the desired fluorescent isoindole. In collaboration with Dr. Thompson, we found that each steroid displays high affinity for the glucocorticoid receptors from rat liver hepatoma tissue culture (HTC) cells and induces the enzyme tyrosine

aminotransferase (TAT). Thus these synthetic steroids show excellent promise for being effective FCA labels for glucocorticoid receptors. Experiments aimed at detecting these postulated, covalent, fluorescent receptor-steroid complexes are in progress.

Following another, more conventional approach, we have tried to develop new reactive functional groups which could be used as electrophilic affinity labeling groups (see also previous reports). A systematic study of the reactivity of α-keto mesylates with various nucleophiles (i.e., carboxylate, -OH, imidazole, $-NH_2$, thiol acid anion and -SH) under mildly basic conditions revealed that α-keto mesylates do not react with imidazole or hydroxyl groups, react extremely slowly (if at all) with carboxylate and primary amines, and react several thousand times faster with thiols and thiol acid anions. The very rapid reaction with thiols occurs only with the dissociated thiolate anion. The addition of a β-hydroxyl group to α-keto mesylates accelerates the reaction with thiolate anions by a factor of 3-12 in acetone but has no effect on reactions run in dimethylformamide. α-Keto mesylates exhibit the same selectivity for thiolate anions, as compared to amines, as do α-keto chlorides. In view of this reactivity and selectivity, the α-keto mesylate appears to be a promising functional group for the electrophilic affinity labeling of biological macromolecules in weakly basic solutions.

Cortisol-21-mesylate (CM) was prepared in hopes of obtaining an alkylating derivative of cortisol. The biological properties of CM were investigated in HTC cells. CM appears to bind to glucocorticoid receptors in cell-free extracts since CM inhibits the specific binding of ^{3}H-dexamethasone. However, in whole cells, CM not only fails to induce the enzyme TAT but also inhibits the induction of TAT by dexamethasone. Thus CM is an anti-glucocorticoid. This is not due to cell death since CM is relatively non-toxic up to concentrations of 10^{-5} M. The concentration of CM needed for half maximal inhibition of TAT induction is an order of magnitude lower than that predicted from the apparent, cell-free affinity of CM for the glucocorticoid receptors of HTC cells, thus suggesting that the cell-free binding data does not reflect an equilibrium situation. In fact the reactive α-keto mesylate group was intentionally incorporated into cortisol in hopes of obtaining a steroid capable of undergoing chemically irreversible reactions. When HTC cells are pre-incubated with either CM or the reversible anti-glucocorticoid progesterone and then washed to remove free steroid, only the CM treated cells fail to show subsequent induction of TAT by dexamethasone. Furthermore, pre-incubation of HTC cell cytosol with CM blocked ∿75% of the subsequent exchange binding of ^{3}H-dexamethasone to glucocorticoid receptor sites. Thus, the actions of CM in whole and broken cells either require an exceptionally long time for reversal or are not reversible. Together, these results indicate that CM is a unique antagonist and could be the first irreversible anti-glucocorticoid in vitro.

We are applying organic chemistry to the study of an important biological molecule(s) - the intracellular steroid hormone receptor(s). A molecular understanding of steroid-receptor interactions is highly desirable, especially since the therapeutically valuable, steroid-specific differences are most likely expressed at this level of steroid hormone action. (S. S. Simons, Jr., D. F. Johnson, M. Pons).

The Development of Chemical Methods and Compounds for the Study of Biological and Medical Problems

The objective of this project is to make contributions to the investigation and solution of basic biological and medical problems by the application of chemical, physical, and biological methods.

The focus of present work is the study of cancer metastases in the mouse. The purposes of this study are: (a) to increase our understanding of the mechanisms operating at the various stages in the formation of metastases, (b) to study the effects of chemicals and biologicals or metastases and their development, and (c) to apply the knowledge obtained thereby to the design and synthesis of organic compound of potential use in inhibiting the formation of metastases or selectively destroying them in human beings. The findings of these studies may also contribute to the study of other biological and medical problems.

The population of cells in most malignant tumors is thought to be heterogeneous. The subpopulation of cells which is most invasive and metastatic may be of special value in the study of the formation of metastases. After fifteen cycles of transplanting cells of lung metastases of the PMT subline of the TU214 sarcoma to muscle in C57B1/6N mice, no increase in metastatic potency of the tumor was found and the study was terminated. The result indicates that the metastatic potency of this tumor cannot be increased by this method and that it may already be maximal. The fact that these cannot be grown in vitro is probably also a manifestation of this high metastatic potency.

A neutral protease has been isolated from cultures of the highly invasive and metastatic TU241-PMT cells freshly isolated from the tumor. This enzyme preferentially degrades type-IV collagen, the principal collagen of basement membrane, and may play a decisive role in the invasiveness and metastatagenicity of the tumor. Studies of metastasis formation in the mouse and measurements of the type-IV collagenolytic activity show a correlation of the metastic potency of B16-F1, B16-F10, B16-BL6, and TV241-PMT cells with this type of enzyme activity. Human leukocytes and MCF-7 human breast carcinoma cells also possess the activity, but mouse fibroblasts and bovine enothelial and aorta smooth muscle cells do not. If this enzyme activity does play a critical role in tumor invasiveness and metastasis formation, it may afford a route to interference with the process of metastasis formation.

A method has been developed for the measurement of basement membrane collagen degradation by tumor cells in vitro. (^{14}C)Proline-labeled type-IV collagen synthesized by EHS sarcoma cells is used to coat 16mm wells of tissue culture clusters. Cells in appropriate media are incubated in the wells and the radioactivity of the fragments of labeled substrate released into the medium is counted. Correlation of metastatic potency with type-IV collagenolytic activity is also indicated using this method. Data obtained by this method also suggest that the mechanism of degradation is local activation of a type-IV collagen-specific enzyme secreted at the cell- substrate interface with no significant phagocytosis of the substrate.

A rabbit antiserum to the type-IV collagenolytic activity has been produced. The antiserum shows slight cytoxicity to TU241-PMT cells in vitro. Such

cells, isolated from the tumor and in single cell suspension, after incubation with the antiserum and intravenous injection produced very few lung metastases in the mouse; whereas, the incubated but untreated control cells were very hihgly metastatic. Work on the preparation of the antibody and its study is in progress. (Calvin M. Foltz, and A. Liotta (NCI)).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19001-08 LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunochemistry and Reaction of Carbohydrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	:	C.P.J. Glaudemans	Chief, Section on Carbohydrates	NIAMDD/LC
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		L.G. Bennett	Staff Fellow	NIAMDD/LC
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COOPERATING UNITS (if any) M. Potter, NCI; R. Feldmann, Computer Center Branch, NIH; M. Bosma, Fox Chase Cancer Institute, Philadelphia, Pa.

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SECTION

Section on Carbohydrates

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Section works on a) elucidation of the structures of polysaccharides which are biologically important; b) the interaction of such oligo- and poly-saccharides with antibodies; and c) the structure and nature of antibodies.

Project Description:

Objectives: Antibodies are isolated from animals and used to study their structure and their interaction with antigens.

Bacterial and fungal antigens are isolated and their structure is elucidated. The mechanism of the immune response is studied.

Methods Employed: Usual techniques of biology and biochemistry are employed, such as affinity chromatography, column chromatography, physicochemical techniques (fluorescence titrations of antibodies) mass-spectroscopy nuclear magnetic resonance, etc.

Major Findings:

(1) The construction of a hypothetical model of the V-region of immunoglobulin J 539 and its interaction with (β1,6 galactosyl-oligosaccharides).

The variable (V) domains of the immunoglobulin light (L) and heavy (H) chains interact to form a structure called the variable fragment (Fv) which contains the antigen binding site. Most of a V-domain structure consists of a framework of antiparallel beta-pleated sheets, but three segments of both the L and H chains are either connecting loops or hairpin bends at the solvent-interface and they may be involved in forming parts of the antigen binding site. Structurally, these segments are the most variable parts of the domain and they can differ from those other V-domains in primary as well as secondary structure. Because these segments potentially contribute antigen-contacting amino acids, they have been called complementarity determining regions (CDR) by Wu and Kabat.

Padlan and Davies established (in 1975) the principle of conformational homology for the V-domain framework. Their finding of the remarkable conformational similarities of two V-region domains, which are derived from species whose last common ancestor existed on earth 15 million years ago, suggested it might be possible to build hypothetical models for other immunoglobulin V-regions from primary structural data. Such a method would utilize the framework of an established structure and then employ various devices for constructing the secondary structure of the differing parts of complementarity determining segments from their primary structures.

In this report we describe a model of the β(1→6)D-galactan-binding myeloma protein J539. In contrast to previously proposed models, the J539 described herein was constructed on a computer display system. We also used the McPC603 as a parent structure but modified it to derive the J539 structure. This was done in two steps. First the J539 amino acid sequences (determined by Rao et al. in 1978 and 1979; and Rudikoff et al. in 1980) were substituted for the corresponding amino acids in McPC603. The

positions of the J539 amino acid side chains were adjusted by a computer program. Second, in three locations the J539 is shorter than McPC603, and these residues in McPC603 were deleted. The peptide chain at the deletion sites was rejoined visually on a computer display system with the least possible perturbation of the polypeptide chain. In this way a less complex structure (J539) was derived from a more complex parent (McPC603). No elements were borrowed from other structures. Both the α-carbon skelton, and a space filling model using characteristic van der Waals radii for each atom type of J539 will be published in the scientific literature (stereophotographs and coordinates are--unfortunately--inappropriate for reproduction in this report. The β1,6 galactosyl trisaccharide ligand was modeled on the computer using both published data from others (Melbert and Rasmussen, 1980) as well as our own laboratory. It was found that the ligand has great conformational flexibility and that the immunoglobulin is probably capable of "freezing out" a conformation it "reads" as suitable for binding.

Galactan binding site. The precise location of the galactan-binding site has not yet been determined in previous studies. There are, however, many observations which suggest its general location. From the work of Wu and Kabat (1970), it appears plausible that the CDR regions of both H and L chains are associated with the binding area. Secondly, many homogeneous anti-β1,6-galactan immunoglobulins studied by us show ligand-induced tryptophanyl fluorescence changes, J539 among them (Jolley et al. 1973). Two solvent-exposed tryptophan residues are located on the frontal surface of the J539 Fv, both in the V_L/V_H crevice, namely Trp 253 (in CDR-1 of V_H (see Table 1 for our numbering systems) and Trp 97 (in CDR-3 of V_L). Thirdly, Navia et al. (1979) have observed that the cavity associated with the hypervariable (CDR) clusters on the frontal surface of J539 is spatially blocked by the constant region of a neighboring symmetry-related molecule in the crystal lattice, and that, indeed, soaking of these crystals in β1,6 galactodiose or β1,6-galactotetarose causes dissolution of the crystal. This suggests that the affinity of the frontal cavity for these ligands leads to binding causing subsequent disruption of the crystal lattice.

Bearing the above in mind we have endeavored to fit the ligands to the general frontal region along the interface between V_H and V_L. We have been guided by a number of considerations: (a) to search for amino acid residues which are generally capable in forming H-bonds. We found these to be in three clusters. The first one in a loop from residue 270-279, and associated with this also is Trp 253 (CDR-2). The second one a cluster consisting of residues 97-99 and 26-39 (CDR-3 and CDR_L-1, and lastly a cluster of residues 323-355 (CDR_H-3) as well as residue 251 (CDR_H-1). (b) In SAPC-10 (another galactan binding IgA) six out of the seven amino acid residues of CDR-3 are different from J539 (Rudikoff, personal communication). The fact that S-10 still binds β1,6 linked D-galactosyl-oligosaccharides (Glaudemans, unpublished results, Rudikoff et al. 1980)

COMPUTER NUMBERING SYSTEM OF McPC603

	V_L		V_H	
	Kabat et al.	This paper	Kabat et al.	This paper
FR1	1-23	1-23	1-30	221-250
CDR1	24-34*	24-40	31-35	251-255
FR2	35-49	41-55	36-49	256-269
CFR2	50-56	56-62	50-65	270-288
FR3	57-88	63-94	66-94	289-320
CDR3	89-97	95-103	95-102	321-331
J	98-107	104-113	103-113	332-341

CDR = complementarity determining region

FR = framework

*Kabat's et al. (1979) numbers 24 to 27 are the same as ours but our positions 28 to 33 are numbered 27A, 27B, 27C, 27D, 27E & 27F in the Kabat System, hence the apparent discrepancy in number of amino acids in this region. Our positions 32 to 38 (or 27E to 32, Kabat et al.) were the ones deleted in J539.

with affinities similar to those of J539 suggests that CDR_H-3 does not partake significantly in the binding. (c) From previous studies (Jolley et al. 1973, 1974; Glaudemans et al. 1975) it appears that the galactooligo (or poly) saccharide makes contact with the protein in such a fashion that O-2, O-3 and O-4 of one sugar ring and O-5 O-1 of the next ring, in such strongest binding subsite (which binds methyl β-D-galactopyranoside) generally has the 6-OH group turned to the solvent, away from the proteins. (d) J539 binds intercatenarily to poly 1,6 linked β-D-galactosyl residues, indicating a groove-type binding region (Roy et al. 1980) rather than a deep, insertion-type open-ended cavity, as in McPC603.

Along those lines we have made two fittings as follows: Model 1-11 was made with 1,6 galactotriose having ϕ, γ and ω angles of 64°, 120° and 55° respectively (as are the published values of gentiobiose, Melberg and Rasmussen, 1980). This mode has the non-reducing terminal galactose near histidine 272, with the second (middle) sugar residue projecting to the solvent and the reducing galactose residue near tyrosine 99. There are preliminary data (Glaudemans, unpublished results) that histidine may be involved in the binding (the K_a increases sharply above the pK of histidine). Also we have found that the second residue in the binding of galactodiose has a high negative enthalpy as well as entropy of binding (Glaudemans, unpublished data). This would indicate immobilization of galactodiose on binding but retention of its hydration and would suggest that only one residue is in close contact with the protein. It is in fact unlikely that the ω angles in the galactotriose will be as high as those reported for gentiobiose. The other fit was therefore made with ϕ, ψ, ω angles of 34°, 173° and 15°. This mode (5-14) has the non-reducing galactose near histidine 272 and the reducing galactose near tyrosine 99. For distances of ligand atoms to selected amino acids in J539, see Tables 6 and 7 (Dr. R. J. Feldmann, Computer Center Branch and Dr. M. Potter, NCI).

(2) Kappa chain joining segments and antibody combining site structural diversity.

Immunoglobulin κ light chains are coded for by at least three distinct gene segments designated variable, joining, and constant. The joining gene codes for the 13 amino acid peptide segment linking the variable and constant regions. This peptide includes the last amino acid (96) in the third complementarity determining region and could thus potentially introduce structural diversity. We have determined the light chain variable region sequences form three myeloma proteins with $\beta(1,6)$galactan binding specificity bringing to six the number of light chains sequenced from proteins demonstrating this specificity. Five of these have Ile at position 96 and the sixth Trp. This substitution appears to be accommodated with no significant change in association constant for a $\beta(1,6)$galactan hapten. Additionally, as many as nine substitutions are found in both light and heavy chain complementarity determining regions between members of this group although only minimal variations in hapten binding affinity are observed. The Ile found at position 96 in five of the κ chains could not be coded for by any of the joining gene nucleotide sequences previously observed and would require a novel nucleotide sequence at the recombination site between variable and joining genes to produce the observed protein structure. Alternatively, there may exist joining gene segments not yet detected (Drs. S. Rudikoff, D. N. Rao and M. Potter, NCI).

DISTANCES IN A° UNITS BETWEEN CERTAIN ATOMS OF GALACTOTRIOSE AND SELECTED AMINOACIDS IN J539

Atoms in Galactotriose(ϕ ψ ω : 34°, 173°, 15°)

J539 Atoms	Terminal											Reducing				
	O1	O2	O3	O4	O5	O6	O1	O2	O3	O4	O5	O1	O2	O3	O4	O5
Ser26 O_γ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ser27 O_γ	+	+	+	+	+	+						8.9	7.1	5.9	6.9	9.0
Ser30 O_γ	+	+	+	+	+	+						+	+	+	+	+
Thr98 $O_{\gamma 1}$	+	+	+	+	+	+						7.5	6.9	6.5	6.2	7.8
Tyr99 OH	+	+	+	+	+	+	8.0	9.6				7.9	6.3	3.9	4.5	7.1
Trp253 Ne_1	7.9	5.2	4.3	6.8	8.2	9.5	+	+	+	+	+	+	+	+	+	+
Glu270 Oe_1	9.3	7.1	6.6	+	9.8	+	+	+	+	+	+	+	+	+	+	+
His272 Ne_2	6.3	4.9	2.1	2.6	5.0	4.8	+	+	+	9.8	9.4	+	+	+	+	+
Ser277 O_γ	+	+	9.0	7.4	8.9	5.6	+	+	+	+	+	+	+	+	+	+
Thr279 $O_{\gamma 1}$	6.5	5.8	3.9	3.6	4.6	2.6	+	+	+	9.2	9.5	+	+	+	+	+

+ = >10

Project No. Z01 AM 19001-08 LC

DISTANCES IN A° UNITS BETWEEN CERTAIN ATOMS OF GALACTOTRIOSE AND SELECTED AMINOACIDS IN J539

Atoms in Galactotriose(ϕ ψ ω : 64°, 120°, 55°)

J539 Atoms	Terminal											Reducing				
	O1	O2	O3	O4	O5	O6	O1	O2	O3	O4	O5	O1	O2	O3	O4	O5
Ser26 O_γ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ser27 O_γ	+	+	+	+	+	+	+	+	+	+	+	+	8.0	+	+	+
Ser30 O_γ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Thr98 $O_{\gamma 1}$	+	+	+	+	+	+	+	+	+	+	+	8.5	6.4	7.8	9.9	+
Tyr99 OH	+	+	+	+	+	+	+	+	+	+	+	+	7.4	7.2	+	+
Trp253 Ne_1	7.0	4.8	3.0	4.9	6.8	7.8	9.8	+	+	+	8.9	9.0	+	+	8.2	8.7
Glu270 Oe_1	8.7	6.3	6.0	8.6	8.6	9.2	+	+	+	+	+	+	+	+	9.2	+
His272 Ne_2	6.8	6.3	4.0	2.4	5.0	3.7	9.9	+	+	9.8	8.5	+	+	+	9.2	+
Ser277 O_γ	+	+	+	9.0	+	7.0	+	+	+	+	+	+	+	+	+	+
Thr279 $O_{\gamma 1}$	7.5	7.5	5.6	4.3	5.3	2.0	+	+	+	+	9.5	+	+	+	+	+

+ = >10

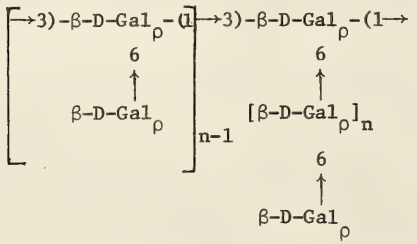
(3) Immunoglobulins with different specificities have similar idiotypes.

Studies in mice on the association and expression of a specific V_H marker (U10-173) with different Ig binding sites showed the following: (1) elevated U10-173 production could be induced by several structurally unrelated carbohydrate antigens, depending on the particular mouse strain chosen; (2) one such antigen, levan, stimulated CWB mice to produce large quantities of anti-levan (1-3 mg/ml) all of which was virtually U10-173$^+$; and (3) unexpectedly, half of the levan-immunized mice produced $\sim$ 2 x more U10-173 than anti-levan. The latter result confirms the original observations of Oudin and Cazenave showing antigen-dependent induction of specific Igs that fail to bind the immunizing antigen. Our results and those of others confirm the original findings of Oudin & Cazenave (1971) who observed Id$^+$ molecules of unrelated binding specificity in the same rabbit as a result of immunization against a single protein antigen. Oudin and Cazenave speculated that the immune cells responsible might have been diversified (stimulated) by a process as yet unknown. Jerne's subsequent conceptualization of such a process (Jerne, 1974) was undoubtedly stimulated in part by the "Oudin-Cazenave enigma". Jerne considered that a single antigenic disturbance in a network of Ids and anti-Ids might result in the following: (a) the production of a specific Id (Id_1) which in turn would block the suppressive effects of cells or molecules with anti-Id_1 specificity; and (b) blockage of the latter would release other Id_1^+ cells from suppression irrespective of their antigen-binding specificities. Whatever the precise mechanism, it is now clear that lymphocytes or antibodies with anti-Id specificity can selectively stimulate or suppress other lymphocytes (as reviewed by Eichmann, 1978) (Drs. E. Enghofer and M. J. Bosma, The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pa.).

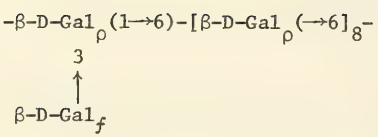
(4) The interaction of two polysaccharides containing β1,6-linked galactopyranosyl residues with two monoclonal antigalactan immunoglobulin Fab' Fragments.

This laboratory has mapped the combining site of murine antigalactan IgA J539 and has reported extensively on the immunochemistry of this general class of proteins. Although D-galactopyranose occurs widely in nature e.g., on glycoproteins and in membranes, the only naturally-occurring polysaccharide originally found to interact with these immunoglobulins was one from larch wood. Since the group of anti-galactan immunoglobulins is quite large, it is to be expected that they would be a counterpart to some closely-related, biologically-relevant antigens. Two such antigens are the galactans isolated from mammalian lung (pneumogalactan [see our earlier reports] and a galactan isolated from the organism Prototheca Zopfii (by Manners et al., in 1973). Apart from the general interest we would have in studying the binding of polysaccharides of mammalian or microbial origins with our anti-galactan IgA's, there is an additional reason for this study. Most immunoglobulins bind to their polysaccharide antigens all along the polysaccharide chain (groove-type site), but there are immunoglobulins which bind only to the terminal carbohydrate residues (cavity-type site) of a polysaccharide (see our last year's report). J539 has been extensively characterized by us, and its amino acid sequence determined. The protein has been crystallized at the NIH by Dr. D. Davies, et al., and further exploration of its combining region is essential. The question of whether this IgA interacts with the terminal residues of galactan chains only, or is capable of binding to any intercatenary stretch of β1,6-D-galactopyranosyl residues as well, has not been resolved so far. The lung galactan is a highly-branched, comb-like polysaccharide (as shown by us before), and the Prototheca Zopfii galactan is an essentially linear galactan. Thus, these two polysaccharides form a good target for the binding studies referred to above.

Antigen-induced fluorencence titration was performed as previously described by us. Protein solutions of OD 0.05 in PBS, pH 7.4, were titrated with increasing amounts of polysaccharide solution, and the change in fluorescence (340 nm) recorded (excitation at 295 nm). The fraction of Fab' sites occupied ($\bar{\nu}$) as a function of the ligand concentration (C) was determined, and a Scatchard plot of $\bar{\nu}/C$ versus $\bar{\nu}$ then yielded the association constant K_a, since $\bar{\nu}/C = K_a - \bar{\nu}K_a$. The structures of the polysaccharides involved are given below. It can be seen that the



Lung galactan



P. Zopfii Galactan

galactan from P. Zopfii contains approximately one branched terminal galactofuransse residue per 9 galactopyranosyl residues, but that the polysaccharide is essentially a linear β(1,6)-galactopyranan. The lung galactan is a highly branched molecule; the backbone is made up of β1, 3-linked D-galactopyranosyl residues, and the side chains are made up of stretches of β1,6-D-galactopyranosyl residues. The twice Smith-degraded larch galactan was shown to be a β1,3-D-galactopyranan (not pictured).

The immunoglobulins J539 Fab' and T601 Fab' interact with the Zopfii and the lung polysaccharides. The binding of the two immunoglobulin Fab' fragments used in these studies to the lung galactan is mediated through the multiple β1,6-linked galactose side chains, since we showed that a β1,3-linked galactan does not bind. From the prior work on the mapping of the combining site J539 by us, it appeared that the protein combiming region is shallow, indicating that it can accommodate the polymer chain segment of an antigen along its surface. However, its capability to bind to intercatenary parts of a polysaccharide antigen remained to be shown. The publication by others of the structure of the galactan from Prototheca Zopfii provided us with a possible test. To our knowledge this galactan is the only naturally-occurring, essentially linear, β1,6-linked galactan known to date. (It is noted that it has single side chains of terminal galactofuranose, but these are spaced some ten sugar residues apart, and it is known that the antibody combining site is capable of accommodating about four sugar residues as we showed many years ago. In addition, we have here shown that synthetic methyl β-D-galactofuranoside does not bind to J539. In Table I we have listed the constants of association (Ka's) for J539 and T601 Fab' fragments with the two polysaccharides investigated. The Ka's in the third column of Table 4 are calculated from the experimental data using for the concentration of polysaccharide

$$C_{ru} = \frac{\text{wt of polysaccharide/liter}}{\text{wt of n anhydrogalactose residues}} \qquad (1)$$

where n = 1.

Polysaccharides do not have a single molecular weight, but have a Gaussian molecular wieght distribution. Thus, if the number average molecular weight is used in the above formula (instead of the weight of n anhydrogalactose residues), the molar concentration of polysaccharide would be obtained. This would lead to the computation of the correct affinity constant only if the entire polysaccharide chain would bind only one immunoglobulin combining site. If the polysaccharide is capable of binding more than one immunoglobulin combining site, one will have to know the "valency" of the other antigen (= the number of sugar residues potentially able to bind one protein combining site). We know that the J539 combining

TABLE 4

Affinity measurements on anti-galactan immunoglobulin fragments with polysaccharides

Fab'	Antigen	K_a per galactose residue	K_a corrected for polysaccharide valency	%ΔF_{max} *	Polysaccharide valency ($\frac{\text{No. galactoses}}{\text{Ig site}}$)
J539	Zopfii galactan	1.7×10^4	2.2×10^5	45	13
J539	Lung galactan	0.8×10^4	1.2×10^5	34	15
T601	Zopfii galactan	1.6×10^4	5.0×10^5	14	31
T601	Lung galactan	0.5×10^4	1.5×10^5	21	30

*% ΔF_{max} is the percentage maximally attainable change in tryptophanyl-fluorescence of the protein as induced by the polysaccharide ligand.

region accommodates maximally about four β1,6-linked residues. Thus, in the expression for the concentration, n would have to be equal to 4. However, we also know from X-ray diffraction studies by others on immunoglobulins that the width of the immunoglobulin combining site is approximately 40 Å. Since each pyranose residue in a β1,6-linked polysaccharide projects approximately 4-6 Å, according to Rees and Scott, the minimum valency of this type of polysaccharide for one immunoglobulin site is n = 7. For that value, the polysaccharide would be completely crowded with bound immunoglobulin. Since this would appear to be unfavorable, it is probable that n > 7, particularly if one realizes that the polysaccharide has no unique conformation in solution and is probably a random coil. The actual valency of the antigen can be measured, and we have determined the valencies of Zopfii galactan and lung galactan by fluorescence titration with J539 and T601 Fab'. From the table it can be seen that these valencies appear higher when the polysaccharides interact with T601 than when they interact with J539 Fab'. We have no explanation for this, but view the values with caution due to the rather large possibilities for error.

Turning again to Table 1, it can be seen that the values of K_a (computed per single galactose residue, third column) are 1.7 and 0.8×10^4 for the essentially linear galactan from Prototheca Zopfii and for the lung galactan, respectively, with J539 Fab'. The lung galactan has short side chains of β1,6-linked galactopyranosyl residues on a backbone of β1,3-linked galactosyl residues.

Since the backbone does not bind to J539 (doubly Smith-degraded larch galactan--a β1,3-linked galactopyranan--does not bind) the observed affinity of the lung galactan is due to the terminal side chains, which account for about 55% of the polysaccharide. Thus, a truer K_a value, based on the affinity per galactose residue which is capable of binding, is 1.5×10^4, and this is close to the K_a value (per galactan residue) of J539 and the Zopfii galactan (1.7×10^4). This similarity alone shows that immunoglobulin J539 can bind intercatenarily to a β1,6D-galactopyranan.

One can also use the values for the "concentration" of repeating stretches of galactose residues (the valency) for the two polysaccharides in question as listed in the last column of Table I. It should be again stressed that those values have a large degree of inaccuracy. Nevertheless, it can be seen from the fourth column (K_a corrected for valency) that these values are of the same order of magnitude as the K_a value for Gal_4, and J539, which is 3.5×10^5.

In addition to the above evidence, it was found that J539 and T601 precipitate with Zopfii galactan and lung galactan. Of these, Zopfii galactan is an essentially linear polysaccharide. Thus, precipitation with J539 and T601 ascites can only come about if the immunoglobulin can bind--not just to the end-galactopyranosyl groups--but to intercatenary residue as well. In that way multiple crosslinks can be formed between immunoglobulin and antigen leading to precipitation. Thus it can be concluded that IgA's J539 and T601 are capable of binding to any part of a β1,6 galactan chain.

(5) Structural studies on the major capsular polysaccharide from cryptococcus bacillisporus serotype B.

Cryptococcus neoformans is the etiologic agent for cryptococcosis, and it produces a large capsule of polysaccharide both in vitro and in vivo. This polysaccharide contains the antigenic determinants for the various serotypes and inhibits phagocytosis by leukocytes and macrophages, and it has therefore been the object of considerable attention.

We have recently reported on the structures of the capsular polysaccharides from C. neoformans serotypes C and D (see last years report). Earlier, others (Bandamer and Danishefsky) described their work on the structure of a capsular polysaccharide from C. neoformans serotype B, but they reported only the sugar composition and the isolation of some oligosaccharides. Thus, a detailed structural analysis of the capsular polysaccharide from serotype B remained to be performed.

Since our last report, serotypes B and C have, on the basis of their biochemical, ecological and genetical differences, been redefined as Cryptococcus bacillisporus serotype B and C, respectively. Below we report our studies on the structure of the preponderant polysaccharide from the capsule of C. bacillisporus serotype B.

The polysaccharide was obtained from a type strain No. 444 isolated from the sputum of a patient with meningitidis. The culture was grown, and the crude polysaccharide isolated.

The purified polysaccharide of C. bacillisporus serotype B was obtained by ion exchange chromatography on DEAE-cellulose using gradient elution. The major component from the column proved to be homogeneous when examined by gel-chromatography on Sepharose 6B and it constituted ∿60% of the crude, capsular material. The K_{av} was 0.13, indicating a molecular weight of $\sim 7 \times 10^5$. This fraction was totally excluded from a column of Sephadex G-200, showing the absence of any contaminant of low molecular weight. The purified polysaccharide appeared homogeneous in immunoelectrophoresis in agar gels versus rabbit anti-type B serum (kindly donated by Dr. J. E. Bennet of the Mycology Section). The material had $[\alpha]_{589}^{20}$ 0° (c 0.25; 1:1 Me_2SO--water), and an O-acetyl content of 10.4%. The ^{1}H-n.m.r. spectrum of the polysaccharide showed a sharp O-acetyl peak at δ2.0 and a broad, single, anomeric peak at δ5.0. The acetate groups are quite labile, as autohydrolysis of the polysaccharide diminished the intensity of the O-acetate peak almost to zero.

Complete hydrolysis of the polysaccharide, followed by chromatography showed the presence of three sugars, having mobilities identical to those of mannose, xylose, and glucuronic acid. This was confirmed by conversion of the sugars into their alditol acetates, followed by g.l.c. in column (a) with inositol hexaacetate as the internal standard. The presence of glucuronic acid in the polysaccharide was confirmed by carboxyl-reduction of the polymer, following which, glucose was detected instead of glucuronic acid. Quantitative analysis showed the presence of mannose, xylose, and glucuronic acid in the ratios of ∿3:3:1. Glucuronic acid was quantitated by a calorimetric

method, and also by g.l.c. (as a derivative of glucose, following hydrolysis of the carboxyl-reduced polysaccharide). Measurement of the optical rotation of the component sugars showed that all three had the D-configuration.

Partial hydrolysis of the polysaccharide yielded one major oligosaccharide; this had a mobility of 0.45 relative to D-glucose in solvent B, and was identified as 2-O-(β-D-glucopyranosyluronic acid)-D-mannose by hydrolysis, by borohydride reduction followed by hydrolysis, and by methylation analysis. The optical rotation, $[\alpha]_{589}^{20}$ -28° (c 0.9, water) indicated a β-linkage, and this was confirmed by the release of D-glucuronic acid from the disaccharide or treatment with β-D-glucosiduronase (E. C. 3.2.1.31:Boehringer--Mannheim) in 0.01 M phosphate buffer, pH 7.3, for 20 h at room temperature.

Oxidation of the acetylated polysaccharide with chromium trioxide for 3 h resulted in the loss of xylose, but the mannose residues appeared to be unaffected, indicating that the xylosyl residues were β-linked, and that the mannose residues were α-linked.

Periodate oxidation of the polysaccharide resulted in the uptake of 1.12 mol of periodate per mol of sugar based on an average molecular weight of 154. Smith degradation of the native polysaccharide resulted inthe total loss of all of the glucosyluronic acid and xylose residues. The periodate-resistant polysaccharide obtained had $[\alpha]_{589}^{20}$+120° (c 0.2; 1:1 Me_2SO--H_2O), and gave only mannose on hydrolysis. Methylation analysis of the Smith-degraded polysaccharide showed the presence of 2,4,6- tri-O-methylmannose as the only hydrolytic product. Therefore, the periodate-resistant core was a (1→3)-linked mannopyranan. The optical rotation, +120°, indicates α-intersaccharidic linkages. Methyl α-D-mannopyranoside and methyl β-D-mannopyranoside, respectively, have $[\alpha]_{589}^{20}$+82° and -70° (c 0.9 and 1.4; 1:1 Me_2So--H_2O).

Methylation of the purified, native polysaccharide, followed by hydrolysis, and analysis of the sugars as their alditol acetates by g.l.c. and g.l.c.--m.s., showed the presence of 2,3,4 tri-O-methylxylose, 4,6 di-O-methylmannose, and 6-O-methylmannose in the molar ratio of ∿2.5:2:1. (The acidic sugar did not elute from the column.) Methylation analysis of the carboxyl-reduced polysaccharide showed the presence of 2,3,4,6-tetra-O-methylglucose, 2,3,4-tri-O-methylxylose, 4,6-O-methylmannose, and 6-O-methylmannose in the molar ratios of ∿1:2:2:1 (see table 5).

Alkaline β-elimination of the methylated polysaccharide, followed by remethylation, and analysis of the alditol acetates, showed the presence of 2,3,4-tri-O-methylxylose, 4,6-di-O-methylmannose, and 2,6-di-O-methylmannose. The absence of 6-O-methylmannose, and the appearance of an equivalent amount of 2,6-di-O-methylmannose, showed that the glucosyluronic acid residues had been linked to O-2 of the mannosyl unites already bearing a substituent on O-4.

From these data, the following structural formula is suggested for the major capsular polysaccharide here investigated:

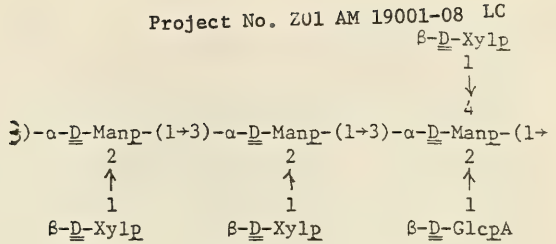


TABLE 5.

METHYLATION ANALYSIS OF THE MAJOR, CAPSULAR POLYSACCHARIDE FROM C. bacillisporus SEROTYPE B

Methylated sugars[a] (alditol acetates)	T[b]	Sample[c] I	II	III	IV
2,3,4-Me_3-Xyl	0.65	2.5	2.0	2.5	-
2,3,4,6-Me_4-Glc	1.00	-	1.0	-	-
2,4,6-Me_3Man	2.10	-	-	-	+
4,6-Me_2-Man[d]	3.34	2.0	1.9	3.0	-
2,6-Me_2-Man[d]	3.38	-	-		-
6-Me-Man	4.50	1.0	1.0	-	-

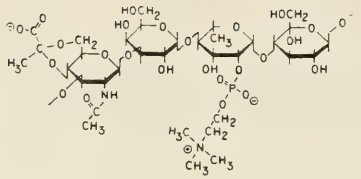
[a] 2,3,4-Me_3-Xyl = 2,3,4-tri-O-methyl-D-xylose, etc. [b] Retention times are relative to that of 1,5 di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol in column (a) at 170°. [c] (I) Methylated polysaccharide; (II) methylated, carboxyl-reduced polysaccharide; (III) methylated, and remethylated, after alkaline degradation; (IV) methylated, Smith-degraded polysaccharide. [d] These two sugars came together as a single peak, and were identified from mixed mass spectra.

The only structural feature not depicted is the location of O-acetyl groups. Estimation of the O-acetyl content by g.l.c. as benzylacetate showed a value of 10.4% and this was supported by the ^{1}H-n.m.r. data. This represents ∿3 O-acetyl groups per repeating unit of seven sugars. We were unsuccessful in an attempt to locate the O-acetyl groups by the method of de Belder and Norrmans, but, from the periodate oxidation, it would appear unlikely that they would occur on the terminal xylosyl or glucosyluronic groups and they are therefore probably located on the mannose core of the polysaccharide, a conclusion that agrees with our immunochemical data (see later). Immunodiffusion in agar gels showed that the polysaccharide of serotype B cross-reacts with both anti type C and anti type D sera. The homologous reaction with the anti type B serum available to us was weak, but this precipition appeared to be undiminished by carboxyl-reduction or carboxyl reduction, followed by O-deacetylation of the type B polysaccharide. It thus appears that the immunodeterminants are groups other than carboxyl or acetate.

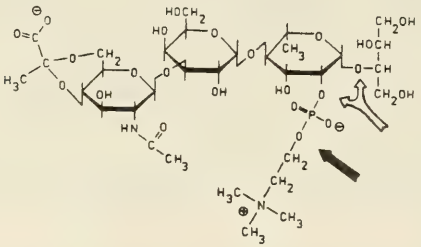
The polysaccharide from serotype B differs substantially from that of C. neoformans serotype D (see last years report). In the latter polysaccharide, one of every three mannose residues in the backbone is unsubstituted, and it does not appear likely that any are doubly branched. Thus, this appears to support the separate species-classification of type D (with type A). (Dr. A. K. Bhattacharjee).

(6) Binding studies with antibodies having phosphorylcholine specificity and fragments derived from their homologous streptococcus pneumoniae type 27 capsular polysaccharide.

This laboratory has a continuing interest in the nature of the interaction of antigens with immunoglobulins. It is particularly useful to look at the various contributions made to the binding of an antigen to its antibody by areas of a different chemical nature that occur in an antigen. Subtle differences may occur in antigens made up entirely of carbohydrate material, such as dextrans or galactans. More varied antigens, such as the capsular polysaccharide from Streptococcus pneumoniae type 27 (S-27) display a variety of antigenic determinants that make it an extremely interesting target for immunochemical studies. This capsular polysaccharide, S-27, consists of four different hexoses (namely, N-acetyl D-glucosamine, D-galactose and L-rhamnose, and D-glucose) and two charged groupings (namely, the ketal of pyruvic acid situated on the glucosamine, and phosphorylcholine (P.C.), situated on the rhamnose) (see Fig. 1). The antigen is susceptible to controlled fragmentation and is thus potentially a superb target for the study of the relative importance of its immunodeterminants. This laboratory has in the past looked at the binding of phosphorylcholine-contaniing antigens with homogeneous immunoglobulins, or with a horse antibody pool. We found that in the case of antibodies to the C-teichoic acid from S. pneumoniae, the phosphorylcholine was a strong determinant, but the polysaccharide backbone of the antigen has a definite capability of binding to the antibody pool. The work we report here is concerned with the interaction of another polysaccharide antigen bearing phosphorylcholine, namely, S-27, and is an attempt at delineating various areas on the antigen capable of interacting with that part of the antibody pool having affinity for phosphorylcholine.



Structure of the capsular polysaccharide from S. pneumoniae type 27.



Structure of the tetrasaccharide isolated from S-27 capsular polysaccharide. Hydrolysis of this tetrasaccharide in 0.01 N NaOH at 100°C cleaves the bond indicated by the dark arrow to yield the decholinated tetrasaccharide. Treatment of the decholinated tetrasaccharide with 49% HF at 4°C cleaves the two bonds indicated by the open double-headed arrow to yield the dephosphorylated trisaccharide.

Periodate oxidation of the S-27 polysaccharide followed by borohydride reduction and mild acid hydrolysis yields a tetrasaccharide of the structure shown. As can be seen in the figure, the phosphorylcholine immunodeterminant of interest is linked to the rhamnose residue at the C-2 position by means of a phosphodiester bond. Selective chemical hydrolysis was used to remove the chloline while leaving the phosphate attached to the polysaccharide backbone. A second hydrolysis was then performed to remove the phosphate group. Studies with these oligosaccharides allowed us to assess the individual contributions of the polysaccharide backbone, the phosphate, and the choline to the overall binding affinity of the whole antigen for anti-S-27 antibody of selected phosphorylcholine specitivity.

A Scatchard plot for two titrations of rabbit anti-P.C. antibody with phosphorylcholine corresponded to a smooth hyperbolic-type curve indicating substantial heterogeneity with respect to binding affinity for P.C. The Sips

heterogeneity coefficient of this antibody pool was determined to be 0.57 for free phosphorylcholine. Other investigators have reported heterogeneity coefficients varying from 0.2 to 1.0 for antibody fractions from serum pools in studies with antibody-dinitrophenyl complexes. Thus it appears that the affinity heterogeneity we observed was in no way unusual. In another titration, however, the data points obtained corresponded more closely to three straight lines of different slopes with a Scatchard plot was constructed. These data suggested three antibody populations, each exhibiting a narrow range of binding affinities within the subpopulation, but with significantly different affinities from the other two subpopulations. For example, the affinity constants determined from the second series in the Scatchard plot shown in Figure 5 were 1.0×10^{3} M^{-1}, 3.3×10^{3} M^{-1}, and 1.2×10^{4} M^{-1}, respectively. Titrations with P.C. and the anti-P.C. antibody pool were repeated several times with the data points sometimes corresponding to a smooth curve with no obvious transition points, and on other occasions the data points showed obvious regions of linearity with a subsequent break into another region of apparently linear points of a different slope. The results of separate titrations are identical for $\bar{\nu}$ values above 0.35 as can be seen in Figure 5. It may be pointed out that an errors in the titration will probably show up in the Scatchard plot for low values of $\bar{\nu}$, since at that stage the amounts of ligand added are small and open to more error. It appears that most of the isolated antibody exhibits affinities in the range from 1×10^{3} M^{-1} to 2×10^{4} M^{-1} when titrated with free phosphorylcholine ligand. Throughout the several titrations with the P.C. ligand, however, the concentration of P.C. required to achieve 50% saturation of the total antibody binding sites was reasonably constant with an average of 6.9×10^{-4} M P.C. (total antibody concentration 2.4×10^{-7} M; (Table 6).

Such binding affinity heterogeneity was not observed in binding experiments where the S-27 tetrasaccharide was the ligand. Titration data with this ligand gave linear Scatchard plots indicative of homogeneous binding affinity. The calculated Sips heterogeneity coefficient from these data was 0.96. Unlike the variations seen in the titrations with P.C., repeated titrations with the S-27 tetrasaccharide gave essentially the same results from one titration to the next. The affinity constant for the intact tetrasaccharide ligand was 7.4×10^{4} M^{-1}, and the concentration of ligand required to achieve 50% saturation of the total binding sites was 1.4×10^{-5} M (total antibody concentration was 2.4×10^{-7} M). The tetrasaccharide ligand was able to achieve 50% saturation of the antibody combining sites at a 50-fold less concentration than was required for P.C. to achieve 50% saturation. Thus, on the average, the anti-P.C. antibody exhibited a 50-fold greater affinity for S-27 tetrasaccharide than for P.C. itself. These data are summarized in Table I.

The obvious range of affinities for P.C. exhibited by heterogeneous serum anti-P.C. was natural and expected, but the apparent binding homogeneity for the S-27 tetrasaccharide was a surprise. One interpretation of these results is that the antibody population with a higher affinity for P.C. is less capable of subsite interaction with the polysaccharide carrier, whereas the population with a lower affinity for P.C. is able to interact with the polysaccharide backbone to a greater extent. Thus, even though antibody-combining subsites may interact to different extents with antigen subsites (choline, phosphate,

Association equilibrium constants, free energy of binding, and concentrations of ligands required to achieve 50% saturation of rabbit anti-P.C. antibody for various ligands

Ligand	Keq (M^{-1})	ΔG (kcal/mole)	Total Ligand Concentration of 50% Saturation (M)[a]
glcNAc $1\rightarrow 3$ gal $1\rightarrow 4$ rha $1\rightarrow 2$ erythritol 4,6-pyruv. 2-P.C. Intact tetrasaccharide	7.4×10^4	−6.6	1.4×10^{-5}
glcNAc $1\rightarrow 3$ gal $1\rightarrow 4$ rha $1\rightarrow 2$ erythritol 4,6-pyruv. 2-PO_4 Decholinated tetrasaccharide	nbd[b]		
glcNAc $1\rightarrow 3$ gal $1\rightarrow 4$ rha 4,6-pyruv. Dephosphorylated trisaccharide	1.4×10^2 to 2.7×10^3	−2.9 to −4.7	
Phosphorylcoline	1.0×10^4 to 1.2×10^4	−4.1 to −5.6	6.9×10^{-4}
Glycerophosphorylcholine	2.6×10^4	−6.0	3.7×10^{-5}

[a] Antibody concentrations 2.4×10^{-7} M.
[b] nbd, No binding detected.

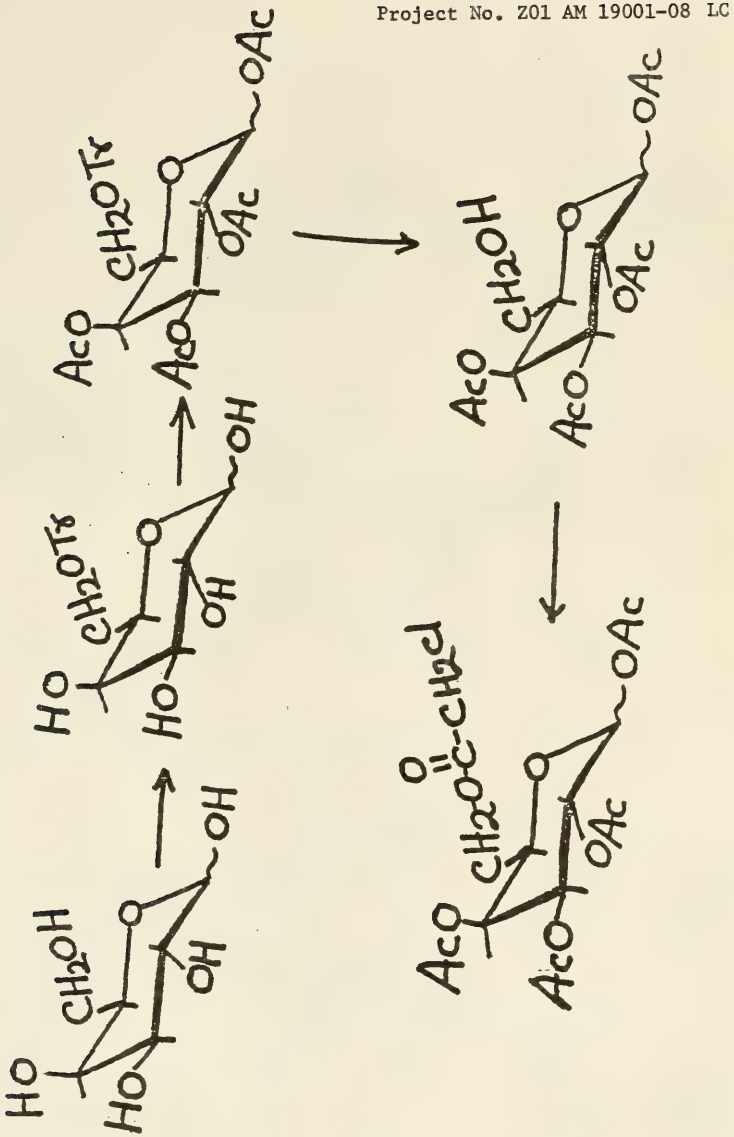
polysaccharide carrier), there is an averaging or compensatory tendency with the net effect of producing a nearly homogeneous binding affinity for the tetrasaccharide within the entire antibody population, in spite of the fact that binding affinity for the P.C. immunodominant moiety is variable. Thus, an immune response may be triggered to any invading antigen of the antibody making up the response has an affinity of ca 7 x 10^4 for any part of the antigenic determinant. (with Dr. L. G. Bennett).

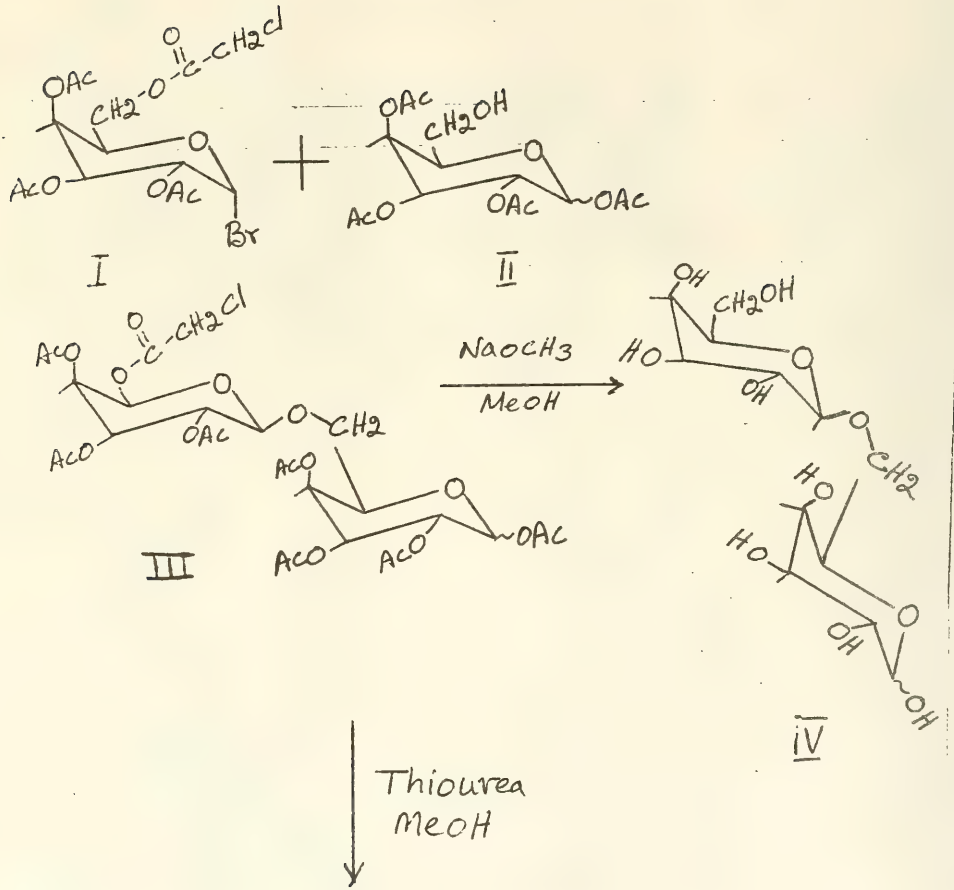
(7) A potential method for the solid-phase synthesis of 1,6-β-linked galactose oligosaccharides.

6-O-β-D-galactopyranosyl-D-galactopyranoside (IV) has been synthesized by the condensation of 6-O-chloroacetyl-1,2,3,4-tetra-o-acetyl-D-galactopyranosyl-bromide (I) with 12,3,4-tetra-o-acetyl-D-galactopyranose (II) by the modified method of Koenigs & Knorr at a yield of 50%. The protected disaccharide (III) was de-chloroacetylated with thiourea in methanol and the resulting compound (V) was condensed with one mole of compound (I) to give the protected trisaccharide (VI) at an approximate yield of 25%. Compound VI on de-O-acetylation gave the free sugar-o-β-D-galactopyranosyl-(1-6)-O-β-D-galacto-pyranosyl(1-6)-O-β-D-galactopyranoside (VII).

The synthesis of compound (I) starting with D-galactopyranose is shown in scheme A and is based on direct tritylation of D-galactose by the method of Karn and Oppenheimer. The overall yield of compound I was 20%.

This method can be adapted for a solid-phase synthesis of β-1,6-linked galactose oligosaccharides.




I
II
III
IV
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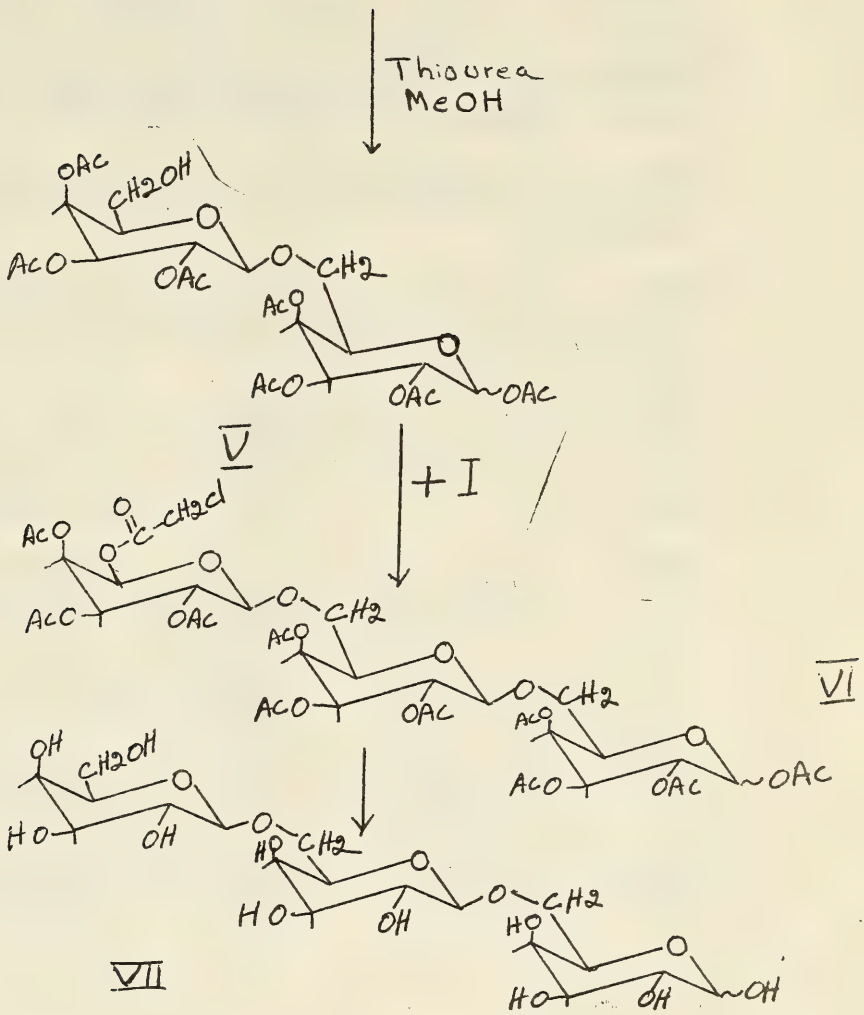
MeOH
Thiourea
MeOH


Thiourea
MeOH
V
+ I
VI
VII

Publications:

Bhattacharjee, A. K., Kwon-Chung, K. J. and Glaudemans, C. P. J.: On the structure of the capsular polysaccharide from Cryptococcus Neoformans Serotype C. II. Molec. Immunol. 16, 531-532 (1979).

Bhattacharjee, A. K., Kwon-Chung, K. J. and Glaudemans, C. P. J.: The structure of the major polysaccharide from Cryptococcus Bacillisporus Serotype B. Carbohyd. Res. 82, 103-111 (1980).

Bennett, L. G. and Glaudemans, C. P. J.: Binding studies with antibodies having phosphorylcholine specificity and fragments derived from their homolgous S. Pneumoniae Type 27 capsular polysaccharide. J. Immunol. 122, 2356-2362 (1979).

Bennett, L. G. and Glaudemans, C. P. J.: Contributions by ionic and steric features of ligands to their binding with phosphorylcholine-specific immunoglobulin A H-8 as determined by fluorescence spectroscopy. Biochemistry 18, 3337-3342 (1979).

Enghofer, E., Bosma, M. and Glaudemans, C. P. J.: Regulation of a small family of V_H-genes. J. Supra Mol. Struct. 9, 3 (1979).

Enghofer, E., Bosma, M. and Glaudemans, C. P. J.: Immunoglobulins with different specificities have similar idiotypes. Molec. Immunol. 16, 1103-111 (1979).

Zidovetzki, R., Blatt, Y., Glaudemans, C. P. J., Manjula, B. N., and Pecht, I.: A common mechanism of hapten binding to immunoglobulins and their heterologous recombinants. Biochemistry 19, 2790-2795 (1980).

Glaudemans, C. P. J. and Bertolini, M.: O-chloroacetates derivatives of sugars as synthetic intermediates. Methods in Carbohyd. Chem. VIII, 271-275 (1980).

Glaudemans, C. P. J. and Jolley, M.: Method for measuring the affinity between carbohydrate ligands and certain proteins. Methods in Carbohyd. Chem. VIII, 145-149 (1980).

Rudikoff, S., Rao, N. D., Glaudemans, C. P. J. and Potter, M.: Kappa chain joining segments antibody combining site diversity. Proc. Nat. Acad. Sci. in press.

Roy, A., Manjula, B. N., and Glaudemans, C. P. J.: The interaction of polysaccharides containing beta 1,6 linked galactopyranosyl residues with two monoclonal immunoglobulin Fab' fragments. Molec. Immunol. in press.

Feldmann, R. J., Potter, M. and Glaudemans, C. P. J.: A hypothetical space-filling model of the V-region of galactan binding immunoglobulin J539. Molec. Immunol. in press.

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PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biological Testing for Antinociceptive Activity, Acute Toxicity and Dependence Liability

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. E. Jacobson	Research Chemist	NIAMDD-LC
OTHER:	E. L. Atwell	Medical Biology Technician	NIAMDD-LC

COOPERATING UNITS (if any)

Department of Pharmacology of the University of Michigan and the Medical College of Virginia, and the Committee on Problems of Drug Dependence, Inc.

LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Animal tests are used to determine the antinociceptive activity of potential analgesic agents. Acute toxicities have, also, been determined on a number of compounds with disparate biological activities. About 40 to 50 compounds have been sent to our cooperating units for further investigation, others have been sent to NCI for screening.

Various mouse assay procedures are utilized for initial exploration of the analgesic potential of molecules. This information is necessary to determine a starting point for their further investigation, at the Medical College of Virginia and the University of Michigan. Thus, compounds which are of interest are examined, under the auspices of the Committee on Problems of Drug Dependence, Inc., in four rodent antinociceptive assays, in a rodent assay for narcotic antagonist activity, and in a series of tests in the rhesus monkey. These are single dose suppression studies, precipitated withdrawal, primary dependence capacity, self-administration and drug discrimination. The first four rhesus monkey studies listed are used to determine the dependence liability of a drug, as compared with morphine. The last test, drug discrimination, is used to attempt to differentiate between compounds which have dependence liability which are more, or less, likely to produce patterns of drug abuse in man. The binding affinity of these compounds to the opiate receptors in rat brain homogenates, electrically stimulated guinea pig ileum and mouse vas deferens, is also determined. These data provide objective information about the pharmacological action and possible side-effects of potential analgesics before human clinical studies are undertaken.

For example, a European group recently asked us to examine an alkaloid, galanthamine, which they appear to be interested in using clinically either as such or in altered form. The compound was found to have been examined by our group in 1956. We reexamined the galanthamine sent to us and found it to have antinociceptive activity in the range between morphine and codeine. Orally, it was found to have morphine-like antinociceptive activity. The compound was tested in the electrically stimulated guinea pig ileum and vas deferens preparations known to have receptors for the opioids. Galanthamine caused contractions of the ileum (like acetylcholine or atropine) with or without stimulation. From these data, and the vas deferens assay, it was concluded that galanthamine does not interact with the opiate receptor. Further, it was found that the compound had considerable toxicity. Its therapeutic ratio was quite low. Obviously, the compound does not appear to be a worthwhile analgesic agent, and should be chemically altered before clinical use is considered.

Acute (7-day) toxicity studies, in mice, of colchicine-like compounds help provide initial information which, coupled with tubulin binding data, allow us to choose compounds for further (NCI) screening.

Publications:

Jacobson, A. E.: Biological Evaluation of Compounds For Their Dependence Liability. IV. Drug Testing Program of the Committee on Problems of Drug Dependence, Inc., 1980. NIDA Research Monograph Series, Washington, D. C., 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19201-06LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Preparation, Receptor Binding Affinity, and Pharmacological Evaluation of N-Cyanoalkyl Opioids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. E. Jacobson	Research Chemist	NIAMDD-LC
OTHER:	K. C. Rice	Research Chemist	NIAMDD-LC
	J. Reden	Guest Worker	NIAMDD-LC
	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC

COOPERATING UNITS (if any)

Laboratory of General and Comparative Biochemistry, NIMH, ADAMHA; Departments of Pharmacology, University of Michigan and Medicinal College of Virginia; Committee on Problems of Drug Dependence, Inc.

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19202-07LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Synthesis and Evaluation of Potential CNS, Antiinflammatory and Anticancer Agents.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. C. Rice	Research Chemist	NIAMDD-LC
OTHER:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC

COOPERATING UNITS (if any)
Medical College of Virginia (E. L. May, L. S. Harris, W. L. Dewey and M. D. Aceto); Pennsylvania State University, York Campus (E. A. Harrison).

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0.1	0.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A simple 3-step synthesis of 3-methyl-4-phenylpyridine a key intermediate in the synthesis of several diverse types of potential pharmaceutical agents is reported. Conversion of this pyridine derivative, which is easily prepared on a larger scale, to a number of the corresponding 2-carboxamides was accomplished. One member of this series was active as a basic, nonsteroidal antiinflammatory agent in the rat adjuvant arthritis test. This work is completed.

Project No. Z01 AM 19202-07 LC

Publications:

E. A. Harrison, Jr., and K. C. Rice: Transformations in the pyridine series. A simple preparation of 3-methyl-4-phenylpyridine and corresponding 2-carboxamides. Heterocycles, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19212-04LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Practical Total Synthesis of Codeine and Other Important Opium Alkaloids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	F. Hsu	Staff Fellow	NIAMDD-LC
	K. C. Rice	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)

University of Wisconsin (D. Pearlman), Oregon State University (J. White)

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NIAMDD, NIH, Bethesda, Maryland 20205

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0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A modified Grewe approach has been employed for total synthesis of (+)-2-hydroxy-4,5-epoxy-6-keto-17-methylmorphinan, an important substance for further defining the role of oxygen functionality on pharmacological activity in the morphinan series. Deoxygenation of this substance by standard methods will provide (+)-3-deoxydihydromorphinone which we prepared earlier in chiral form from natural morphine. Selective oxidation of this substance at the 3-position with chemical and microbial agents is being studied as a potential route to codeine and other opium alkaloids.

PHS-6040

The interesting pharmacological profiles we observed earlier for a number of semisynthetic 3-deoxyopioids and the possibility of utilization of certain of these compounds as close or direct precursors of important opium alkaloids prompted us to examine totally synthetic routes to 3-deoxyopioids. Along these lines, an efficient synthesis of 2,4-dihydroxy-6-ketomorphinan has been developed using a Grewe-type approach. Conditions were not found for selective removal of the hydroxyl function at C-2 in this system. However, closure of the oxide bridge could be accomplished, preferably with the N-formyl derivative, to afford the corresponding monophenolic 1-bromo-2-hydroxy-4,5-epoxy derivative. Hydrolysis of the N-formyl substitutent, and hydrogenation in the presence of formaldehyde gave (+)-4,5-epoxy-2-hydroxy-17-methylmorphinan-6-one. Pharmacological examination of this key substance and the corresponding methyl ether is in progress to provide additional insight into the role of oxygen functionality on biological activity. Furthermore, removal of the phenolic hydroxy group by standard methods will afford (+)-3-deoxydihydromorphinone, which we prepared earlier in chiral form from natural morphine. The problem of selective introduction of an oxygen function at C-3 is currently being addressed as a novel entry into the natural opioid series.

Publication:

Hsu, F.-L., Jacobson, A. E., Rice, Kenner C, and Brossi, A.: Partial Synthesis of 3-Deoxydihydromorphine from (-)-4-Hydroxy-6-keto-N-methylmorphinan, Heterocycles, 13, 259-261 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19214-04LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Direct and Sequential Removal of Phenolic Oxygen Functionalities in Alkaloids.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	J. Minamikawa	Research Fellow	NIAMDD-LC
	K. C. Rice	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)

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NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Temporarily discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19216-04LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Molecular Features of Colchicine Associated with Antimitotic and Antigout Activity; N-Hydroxyacetylated Colchicine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	M. Rösner	Visiting Fellow	NIAMDD-LC
	A. E. Jacobson	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)
Istituto di Sanita, Rome (M. Iorio), Laboratory of Environmental Biophys. (C. F. Chignell)

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NIAMDD, NIH, Bethesda, Maryland 20205

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1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A series of compounds based on the natural alkaloid colchicine were examined in the NCI P388 lymphocytic leukemia screen in mice and for their affinity to tubulin. The toxicities of the compounds were examined and compared. In general, a direct relationship between tubulin binding and in vivo activity was not found, but the binding affinity of new colchicine-like molecules to tubulin could be utilized in the search for new anti-cancer drugs. The combined pharmacological information has indicated that molecules formed by a triplet of chemical changes in colchicine may result in potent compounds with lessened toxicity.

Congeners of colchicine, containing an N-hydroxyacetyl group instead of the usual N-acetyl group, have been found among natural products. The synthesis of one of them, colchifoline, has been accomplished.

PHS-6040
(Rev. 10-76)

We have found that N-formyldemecolcine retains, essentially, the in vivo potency of natural colchicine in the NCI P388 lymphocytic leukemia screen in mice, most of the affinity of the parent molecule in tubulin binding, and is less toxic. N-Trifluoroacetyldemecolcine and 3-demethylcolchicine are also considerably less toxic than colchicine, have about the same affinity as N-formyldemecolcine in tubulin binding but are, however, somewhat less potent in the P388 assay than colchicine. The conversion of the secondary amino group of colchicine at C-7, into a tertiary amine, combined with a modification of the acetyl group has resulted in P388 active compounds with decreased toxicity. 3-Demethylcolchicine, alone among the aromatic ring A demethylated compounds, retains considerable potency in the P388 assay. The 1-methoxy, and 2-methoxy-groups are, apparently, essential for both in vivo potency and tubulin binding. It is possible that there is a hydrogen-bonding moiety on the receptor, at some distance from the 1- and 2-methoxy groups. Conversion of these methoxy groups to the phenolic compound could cause the molecule to change its orientation sufficiently to allow H-bonding between that phenolic group, and some moiety in tubulin, and remove the ring C oxygen atoms from their critical binding area, lowering overall affinity in tubulin binding. A direct relationship between tubulin binding and P388 potency can be observed in the 3-, 2-, and 1-demethyl series. Their in vitro and in vivo potencies, are also directly related, qualitatively, to in vivo toxicities; as the potency and tubulin binding affinity increase, so does the toxicity. Thus, a combination of changes on two sites of the colchicine molecule does not greatly alter potency, compared with colchicine, in the P388 screen and which appear to decrease the parent molecule's toxicity. That is, the change of the 3-methoxy group of colchicine to the 3-phenol, and the change in the type of amide (secondary vs tertiary) and substituent on the amide. Conversion of the ring C methoxy group to a thiol ether does not lessen tubulin binding. Also, thiocolchicine is at least as effective in vivo in the PS388 screen as is colchicine itself. Its toxicity is known to be greater than colchicine in this assay, however. The interchange of oxygen for sulfur in the third site of the colchicine molecule would not appear to be as advantageous as changes at C-3 in ring A or C-7 in ring B if a major concern was decreased toxicity.

All other changes in the colchicine molecule appear to be disadvantageous. Conversion to the isocolchicine or isodemecolcine structure in ring C, completely abolished activity. A change in the double-bond pattern, as in precolchicine eliminated its affinity for tubulin binding. Conversion of the amide moiety to a secondary or tertiary amine caused a considerable loss of biological activity, or its total loss.

It was observed that tubulin binding and in vivo potency in the P388 system are not directly related. Thus, demecolcine and the Δ^6-isomer B bind as well as colchicine in the tubulin assay but are one or two orders of magnitude less potent than colchicine in the P388 assay. However, the tubulin assay may be very useful if looked at as follows.

Compounds which show less than ca. 50% tubulin binding always appear to be inactive, or almost so, in the P388 screen. Those compounds which show an affinity between the 50 and 60% levels may have some in vivo activity and compounds with affinity greater than 60% are worthy candidates for the P388 assay.

The colchicine-like P388 activity of N-formyldemecolcine, and its lowered toxicity, may be indicative of the fact that a colchicine-like molecule might be prepared which could be clinically useful in the future. This compound is the result of two modifications in the C-7 position of ring B. It is possible that a third change, conversion of the 3-methoxy group to a 3-phenolic moiety when coupled with the previous changes, will produce a compound with sufficient in vivo potency and markedly lowered toxicity. Synthesis of this type of compound is in progress.

Recently some novel acylated colchicine congeners have been isolated from plants and have been found as minor impurities in commercial samples of colchicine. In order to study the effect of N-hydroxyacetylated colchicine congeners on tubulin binding, with regard to toxicity and as antitumor agents in vivo, colchifoline, found in commercial samples of colchicine, was synthesized by an efficient procedure.

Phenolic colchicine congeners: Experimental conditions were found which allow the preparation of 1- and 2-demethylcolchicine from colchicine and 3-demethylcolchicine from colchicoside. All three monophenols were evaluated as potential antitumor agents.

Phenolic colchicine congeners were found among natural products and their evaluation as potential antitumor agents is much desired. The yield of these compounds by isolation from plant sources or microbial fermentation of colchicine is too small to provide a source. Tedious experimentation has shown that the 1-methoxy group of colchicine can be cleaved with the procedure published by Bladé-Font. The 2-methoxy group can be cleaved with concentrated sulfuric acid to afford the wanted phenol in 50% yield. Similarly the glycoside linkage of colchicoside can be cleaved with 85% phosphoric acid at room temperature to afford this important phenol in high yield. From the 3 phenols the 3-demethylcolchicine showed the best therapeutic index in the P388 assay, and it will be further modified.

Publications:

1. M. Rösner, H.-G. Capraro, A. E. Jacobson, L. Atwell, A. Brossi, R. H. Sik, C. F. Chignell, and M. Iorio: Biological Effects of Modified Colchicines. Improved Preparation of 2-Demethylcolchicine, 3-Demethylcolchicine, and (+)-Colchicine, J. Med. Chem., in press.

2. M. A. Iorio, A. Mazzeo-Farina, G. Cavina, L. Boniforti and A. Brossi: Minor Alkaloids in Commercial Samples of Colchicine, Heterocycles 14, 625-630 (1980).

3. M. A. Iorio, M. Molinari and A. Brossi: Synthesis of Colchifoline from Deacetylcolchiceine, Canad. J. of Chem. (1980), in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19218-02 LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

N-Acetylated Pyrimethamine as Antimalarials

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	N. Acton	Guest Worker	NIAMDD-LC

COOPERATING UNITS (if any)

Divison of Medicinal Chemistry, Walter Reed Army Institute of Research (Dr. T. Sweeney).

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NIAMDD, NIH, Bethesda, Maryland

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0.5	0.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is now under project number Z01 Am 19232-01LC

FORM 2590
Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19219-03LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Synthesis of 3-Deoxymorphine and Dihydro Congeners, and their Pharmacological Evaluation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	A. E. Jacobson	Research Chemist	NIAMDD-LC
	K. C. Rice	Research Chemist	NIAMDD-LC
	F. Hsu	Staff Fellow	NIAMDD-LC
	M. Rozwadowska	Visiting Scientist	NIAMDD-LC

COOPERATING UNITS (if any)

School of Pharmacy, University of Mississippi, University, Mississippi (C. D. Hufford)

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Laboratory of Chemistry

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Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Study of a number of chemical transformations of (-)-3-deoxydihydromorphinone, which is now readily available from natural (-)-morphine as a result of our earlier work, has provided much insight into the chemistry of 3-deoxyopioids that we required for our program on total synthesis in this area. (Project Z01 AM 19212-03LC). Pharmacological evaluation of a number of compounds generated in this work and by total synthesis is in progress, or if complete, is reported under project number Z01 AM 19228-02LC.

PHS-6040

(-)-4-Hydroxy-N-formylmorphinan-6-one was readily prepared in 70% overall yield from 3-deoxydihydromorphinone by treatment with 2,2,2-trichloroethyl-chloroformate, reductive cleavage of the carbamate function and oxide bridge concomitantly, followed by N-formylation. Bromination of this intermediate afforded the 1-bromo-4-hydroxy congener or the corresponding 4,5-epoxy derivative, depending on the conditons used. These intermediates which were shown to undergo a number of other transformations, could be converted by standard methods to (-)-3-deoxydihydromorphinone. Thus, (-)-4-hydroxy-N-formylmorphinan-6-one is a versatile intermediate, well suited for synthesis of (-)-4-hydroxy-morphinans and 3-deoxyopioids.

Publications:

Rozwadowska, M. D., Hsu, F.-L, Jacobson, A. E., Rice, K. C., Brossi, A.: (-)-4-Hydroxy-N-formylmorphinan-6-one, A Versatile Intermediate for the Synthesis of 3-Deoxyopioids. Can. J. Chem., in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19222-03LC

PERIOD COVERED

October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Retinylidine 1,3-Diketones as Potential Antitumor Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	N. Acton	Guest Worker	NIAMDD-LC

COOPERATING UNITS (if any)

M. Sporn, NIH Cancer Institute

LAB/BRANCH

~~Laboratory of Chemistry~~

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

~~NIAMDD, NIH, Bethesda, Maryland 20205~~

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.9	0.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Ring oxygenated analogs of retinylidene dimedone (RD) have been prepared. RD analogs containing a terminal 1,3-diketone moiety are cleaved by aqueous methylamine. The rate of this retro aldol cleavage parallels the in-vivo activity of these compounds in the hamster tracheal organ culture test, suggesting that they act as prodrugs of retinoic acid.

PHS-6040

Ring oxygenated analogs of RD were prepared but found less active in the hamster tracheal organ assay of Dr. Sporn. RD, analogs and some of these oxygenated derivatives are being cleaved with aqueous methylamine into the corresponding aldehyde and dimedone, or corresponding 1,3-diketones. The rate of this cleavage parallels the observed in vivo activity, suggesting that the class of retinal derived 1,3-diketones acts as a prodrug of retinoic acid. The cleavage of the oxygenated analogs of RD afforded the oxygenated derivatives of retinal in a high state of chemical purity, suggesting that purification of retinal derived compounds might be attempted through the dimedone derivative.

Publications:

1. Acton, N., Brossi, A., Newton, D. L. and Sporn, M. B.: Prophylactic Antitumor Activity of Retinylidene-1,3-diketones: J. Med. Chem. in press, 1980.

2. Acton, N. and Brossi, A.: Oxydation Products of Retinylidene Dimedone: Helv. Chim. Acta., in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19223-03LC

PERIOD COVERED

October 1, 1979 to September 30 1980

TITLE OF PROJECT (80 characters or less)

Tetrahydropapaveroline

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	C. P. Mak	Visiting Fellow	NIAMDD-LC
	A. E. Jacobson	Research Chemist	NIAMDD-LC
	J. Reden	Guest Worker	NIAMDD-LC
	K. C. Rice	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)

P. Skolnick, Y. Nimitkitpaisan, and J. Daly, Lab. of Bioorganic Chemistry, NIH

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(+)-Tetrahydropapaveroline, its optical isomers, and related compounds were studied for their binding to β-adrenergic, dopaminergic, and α-adrenergic receptors from rat cerebral cortex. The related compounds were (+)-trimetoquinol; (+)-, (S)-(-), and (R)-(+)-N-norreticuline, papaveroline, 6,7-dihydroxy-1-(4-hydroxy-3-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid and 6,7-dihydroxy-1-(3,4-dihydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid, 6,7-dihydroxy-.-(4-hydroxy-3-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline, 1-(3,4-dihydroxybenzyl)-7-hydroxy-6-methoxy-1,2,3,4-tetrahydroisoquinoline, 6,7-dihydroxy-1-(6-bromo-3,4-dihydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline, norlaudanosine; 2,3,9,10-tetraacetoxydibenzo[bg]-pyrrocoline, 2,3,8,9-tetrahydroxy-isopavinan and 2,3,8,9-tetrahydroxypavinan. THP has been considered a "mammalian alkaloid"; its endogeneous formation in mammals prompted us to examine its interactions with mammalian receptors and to examine compounds related to THP. (+)-Trimetoquinol was among the most potent of these compounds in inhibiting binding to β-adrenergic receptors, and 6'-Br-THP was more potent than dopamine in the α-adrenergic assay and equipotent to it in the dopaminergic receptor assay.

All of the active compounds which bind as well as or better than (+)-norepinephrine in the β-adrenergic receptor assay system are conformationally mobile, or flexible, compounds. Their benzyl groups can twist around the C-1 position of the tetrahydroisoquinoline moiety. This conformational flexibility, and the presence of a secondary amine, appears to enable these molecules to interact with the β-adrenergic receptors. Tertiary amines have been noted to be inactive in a β-adrenergic binding system. An aromatic isoquinoline was inactive, as were other compounds which are multiring, conformationally inflexible molecules. Since it is very unlikely that the O-methyl ethers can be converted to catechols or that the catechols can be O-methylated under the in vitro assay conditions, it is apparent that the catechol moiety is not essential for this interaction. Obviously, however, the most potent of the studied compounds in binding to the β-adrenergic system is (+)-trimethoquinol, which has a catechol group. Thus, catechol groups may aid in the interaction. The carboxylic acid derivatives were very much less active than the other tetrahydroisoquinolines. It is possible that these compounds are much less conformationally mobile than the active tetrahydroisoquinolines. Similar carboxylic acids have been noted to be geometrically fixed, with the benzylic portion of the molecule twisted away from the tetrahydroisoquinoline moiety. The carboxylic acid moiety apparently lies close to the tetrahydroisoquinoline C-8 proton, which has been observed, in the NMR, to be deshielded, presumably due to the influence of the CO_2H group. Thus, it is possible that the conformation of the active compounds responsible for binding in this assay system is one in which the aromatic part of the benzylic group is in close proximity to the aromatic ring of the tetrahydroisoquinoline moiety. Only the secondary amines and their active enantiomers, can attain that conformation.

The structure-activity relationship for binding in the α-adrenergic and dopaminergic assay systems appears similar to that in the β-adrenergic assay system. However, virtually all of the compounds in the β-adrenergic assay system are at least an order of magnitude less potent in the dopaminergic and α-adrenergic assay systems. The only exceptions were (-)-N-norreticuline, which was equipotent in the β-adrenergic and dopaminergic receptor assays, and the 6'-Br-THP, which was very much more potent in the a-adrenergic assay than in the β-adrenergic assay and somewhat more potent in the α-adrenergic assay than in the dopaminergic assay. Steroselectivity for the optical isomers of THP and the N-norreticulines appears less for the α-adrenergic receptors than for either the β-adrenergic or dopaminergic receptors.

Publications:

A. Brossi, K. C. Rice, C. P. Mak, J. Reden, A. E. Jacobson, Y. Nimitkitpaisan, P. Skolnick, and J. Daly: Mammalian Alkaloids. 8 . Synthesis and Biological Effects of Tetrahydropapaveroline Related 1-Benzyltetrahydroisoquinolines, J. Med. Chem., 23, 648-652 (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19224-02LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Microbiological oxidation of Morphines and Morphinans.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	A. E. Jacobson	Research Chemist	NIAMDD-LC
	F. Hsu	Staff Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

Waksman Institute of Microbiology, Rutgers University (C.P. Schaffner)

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Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

a microbial or fungal system capable of the conversion of 3-deoxyopioids to their phenolic hydroxyl derivative is being sought. This conversion may provide a simpler route to the natural alkaloids, utilizing chemical and microbiological transformations.

PHS-6040
(Rev. 10-76)

Chemical procedures have provided a practical route to 3-deoxydihydro-morphine. Microbiological conversion of this type of compound would complete the overall transition from simple starting materials to morphine-like alkaloids.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19225-02LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Investigation of an unusual 7-ketodihydromorphinan

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	J. Minamikawa	Research Fellow	NIAMDD-LC
	K. C. Rice	Research Chemist	NIAMDD-LC
	A. E. Jacobson	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)
Dr. J. V. Silverton, Lab. of Chemistry, NHLI; Williams, Hoffman-LaRoche, Inc., N. J.

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Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Work completed.

PHS-6040
(Rev. 10-76)

Publications:

1) Minamikawa, J., Rice, K., Jacobson, A. E., Brossi, A., Williams, T. Silverton, J.: Studies in the (+)-Morphinan Series. VIII. Unusual crystallographic and tautomeric properties of 7-keto-3-methoxy-4-hydroxy-5,6-dehydromorphinan; An Interlacing Double Helix, J. Org. Chem., 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19226-02LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Synthesis and Pharmacology of Irreversible Inhibitors of the Benzodiazepine Receptor.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. C. Rice	Research Chemist	NIAMDD-LC
OTHER:	P. Skolnick	Pharmacologist	NIAMDD-LBC
	E. Williams	Staff Fellow	NIAMDD-LBC

COOPERATING UNITS (if any)

J. Tallman, NIMH-BP, S. Paul, NIMH-CP

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Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several benzodiazepines containing reactive functional groups were synthesized as specific, irreversible inhibitors of the benzodiazepine receptor in order to further study and characterize these receptors, through which the pharmacological properties of benzodiazepines are expressed. Binding studies using the alkylating benzodiazepine, kenazepine, strongly suggest the existence of several populations of benzodiazepine (BZ) receptors in the rat CNS. Kenazepine reacts non-competitively and irreversibly with some receptors and competitively (reversibly) with others. Cerebellum contains the largest proportion (ca. 80%) of the non-competitive type, while hippocampus and cortex contain a preponderance of competitive type receptors (ca. 80% and 50%, respectively). The Hill coefficients for kenazepine are near 0.7 in cortex and cerebellum, and near unity in dorsal hippocampus. Different populations of BZ receptors may mediate different physiologic and pharmacologic effects in vivo.

SmS-6040
(Rev. 10-76)

The benzodiazepines are among the most widely prescribed drugs worldwide, and have recently been shown to exert their pharmacological actions by reversible interaction with high affinity binding sites in the mammalian central nervous system. In order to further characterize these receptors and to more clearly define the mechanism of action of these drugs, we have designed and synthesized benzodiazepines which act as specific, irreversible inhibitors of the receptors. These inhibitors function by specific interaction with the receptor, followed by covalent bond formation between a reactive functional group in the benzodiazepine and suitable functionality in the receptor.

Receptors irreversibly labeled (identified) in this manner are differentiated from other proteins present in brain tissue and are thus much more amenable to study than the unlabeled counterparts. Irazepine, a benzodiazepine containing the reactive isothiocyanate moiety, was shown earlier to be the first example of a specific irreversible inhibitor of benzodiazepine receptor. Recent work with the alkylating benzodiazepine kenazepine has shown the existence of two types of benzodiazepine receptors which may function separately to regulate the different central effects of benzodiazepines. Further work along these lines is in progress and includes attempts to isolate and purify the receptor previously solublized (J. Tallman).

Publication: Williams, Evan, Rice, Kenner, C.; Paul, Steven, M., and Skolnick, Phil: Heterogeneity of benzodiazepine receptors in the central nervous system demonstrated with kenazepine, an alkylating benzodiazepine. J. Neurochem., in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19227-02LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Synthesis of Racemic and Optically Active Pavinan and Isopavinan Alkaloids from N-Norreticulines.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	K. C. Rice	Research Chemist	NIAMDD-LC
	J. Reden	Visiting Scientist	NIAMDD-LC
	W. Ripka	Guest Worker	NIAMDD-LC

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES x☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project discontinued

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19228-02LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Conversion of dihydromorphine to 4-hydroxy-6-ketomorphinan and the ether opioids.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	F. Hsu	Staff, Fellow	NIAMDD-LC
	A. E. Jacobson	Research Chemist	NIAMDD-LC
	K. C. Rice	Research Chemist	NIAMDD-LC
	M. Rozwadowska	Visiting Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

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Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A series of compounds, based on 4-methoxy-6-ketomorphinan, and on 4-methoxymorphinan were synthesized and biologically evaluated for their antinociceptive activity. The biological activity of these compounds were compared with the 3-deoxyopioids. The N-H, N-formyl and N-methyl analogs were examined. The N-methyl-4-methoxy compounds were found to be more potent antinociceptive agents than the comparable 4-hydroxy molecules. Thus, 4-methoxy-6-ketomorphinan was found to be three times more potent than morphine as an antinociceptive, as was 3,4-dimethoxymorphinan.

PHS-6040
(Rev. 10-76)

It was found, from the accumulated biological data, that in this series the 3-hydroxyl group is unnecessary for antinociceptive activity. We noted, in comparing molecularly comparable compounds, that compounds with an ether bridge (the 4,5-epoxy group) are more potent than those with a 4-hydroxy group (ring opened). As anticipated, within a comparable class, the following order of potency was observed: $N\text{-}CH_3 > N\text{-}H > N\text{-}CHO$. The more potent compounds will, in furture research, be converted to the agonist-antagonist type of molecule and examined in a wide variety of biological screens. It is of theoretical interest to note that methoxy compounds, generally, do not bind well to the opiate receptor. Presumably, their ability to more easily traverse the blood-brain barrier could account for their in vivo activity. However, the well-known argument that the in vivo activity of codeine is due to in situ cleavage to morphine would not pertain in this series where, if the ether was biologically cleaved to the phenolic compound, the metabolite is considerably less potent as an antinociceptive.

Publication:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19229-01LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Metabolism of 3,6-Dideoxydihydromorphine in the Rhesus Monkey.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. E. Jacobson	Research Chemist	NIAMDD-LC
OTHER:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
	N. Whittaker	Chemist	NIAMDD-LC

COOPERATING UNITS (if any)

Research Institute of Pharmaceutical Sciences, University of Mississippi (Drs. J. A. Bedford, M. A. El Sohly).

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NIAMDD, NIH, Bethesda, Maryland 20205

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0.3	0.3	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our finding that 3,6-dideoxydihydromorphine had antinociceptive activity equivalent to morphine and that, in the rhesus monkey, its pharmacological actions also paralleled those of morphine (e.g.-physical dependence liability), led us to study the metabolism of the compound. It was, a prior, possible that the compound could be hydroxylated in vivo and the resultant phenolic compound could be responsible for the observed pharmacological effects. The study has shown, thus far, that 3,6-dideoxydihydromorphine is not metabolized to a phenolic compound. Thus, contrary to previous assertions in the literature, the phenolic hydroxyl group in opioids is not essential for either antinociception or physical dependence.

PHS-6040
(Rev. 10-76)

The metabolites of 3,6-dideoxydihydromorphine, obtained from the urine of rhesus monkeys, were examined by a variety of analytical techniques including thin layer chromatography, gas chromatography, and high resolution mass spectrometry. THe main metabolite found was 3,6-dideoxydihydronormorphine, the result of N-demethylation. Since few N-demethyl opioids have antinociceptive activity (even after intracerebroventricular injection), and none have been noted to have potency greater than the N-methyl parent, it can be concluded that 3,6-dideoxydihydromorphine, itself, is responsible for the previously observed pharmacological activities (antinociception and physical dependence). Since it does not interact particularly effectively (low binding affinity) with the opiate receptor in vitro, its greater lipophilicity (as compared with morphine) must have a considerable bearing on its activity.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM19230-01LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Perhydrohistrionicotoxines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	K. Takahashi	Visiting Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

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SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.1	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A practical synthesis of (+)-perhydrohistrionicotoxine has been developed on the basis of published procedures. It will now for the first time be possible, to study this toxine and analogs, prepared in the course of this investigation, in great detail.

PHS-6040
Rev. 10-79)

Perhydrohistrionicotoxine obtained from natural histrionicotoxine by hydrogenation has extremely interesting biochemical properties and is a target compound for studying the ionic channel of the acetylcholine receptor. The naturally derived material is hard to come by and has until recently almost exclusively been prepared from Columbian frogs. Also there are several published syntheses of (±) perhydrohistrionicotoxin, none has been developed on a scale where it could have been considered adequate for preparing sufficient quantities of material needed for biological testing. Investigation of the published syntheses have now led to a variation which afforded (±)-perhydrohistrionicotoxin in sufficient quantity. Analogs have been prepared by the same route. The testing of intermediates has shown that the alkyl group belonging to the spiropiperidine unit is not needed for biological activity since the desamyl compound is already highly active. With the material now at hand, optical resolution and investigation of the optical isomers as well as manipulation of the oxygen function is contemplated.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19231-01LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Racemization of R-(+)- and S-(-)-Norreticuline

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	K. Rice	Research Chemist	NIAMDD-LC
	P. Buchs	Visiting Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Efficient chemical conversion of S-(-)-norreticuline into its R-enantiomer is mandatory for a practical conversion into morphine and related opioids. Several avenues are being investigated.

PHS-6040
(Rev. 10-76)

Only S-(-)-reticuline, obtained from R-(+)-norreticuline, is utilized by the opium poppy for the biosynthesis of morphine, and only R-(+)-norreticuline or its derivatives, afford opioid congeners by chemical synthesis. The optical isomer S-(-)-norreticuline on the other hand provides the entry into the series of (+)-opioids. Asymmetric syntheses leading to only one of the two enantiomers or conversion of one enantiomer into the other via the racemate are possibilities to solve this problem. Chemical oxidation of the phenolic norreticulines is complicated by the fact that it may easily lead to novel structures. Aromatization of the optically active norreticulines to 3',7-demethylpapaverine over Pd-catalyst in decalin has been accomplished in 60% yield and catalytic reduction of this aromatic compound affords racemic material. The practicality of this 2-step conversion is being further explored.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19232-01LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Conversion of Antimalarial Amino Alcohols into Aziridines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	M. Rösner	Guest Scientist	NIAMDD-LC

COOPERATING UNITS (if any)

J. V. Silverton, NIH, National Institute of Heart, Lung and Blood, Walter Reed Army Medical Center

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SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The antimalarial mefloquine was converted into an aziridine compound. The structure of the latter was secured by X-ray crystallography. Chemical reactions of the novel aziridine afforded a variety of substituted mefloquines which are being evaluated as antimalarials.

PHS-6040
(Rev. 10-78)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19233-01LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Total Synthesis of Opioids via Dihydrothebainone and Derivatives

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. C. Rice	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)

None

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SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Racemic dihydrothebainone, nordihydrocodeinone and dihydrocodeinone were synthesized in high overall yield from 3-methoxyphenethylamine, via the key intermediate (±)-1-bromonordihydrothebainone; the route utilized unprotected phenolic intermediates, involved directed Grewe-type cyclization and for nordihydrocodeinone and dihydrocodeinone exploited novel oxide bridge closure in the N-nor series. Practical total syntheses of codeine and other opium derivatives now appear available in view of these results and those of others.

PHS-2043
(Rev. 10-76)

Natural (-)-codeine continues to occupy a position of central importance among the medically valuable derivatives of the opium poppy as the most frequently prescribed analgesic-antitussive agent worldwide. Since the first total synthesis of (-)-codeine and (-)-morphine other successful routes including Grewe-type and biomimetic approaches, have appeared. However, a practical total synthesis of these drugs has remained elusive. These and continuing efforts, together with possible shortages, underscore the desirability of securing a route which could render licit production of medical opiates independent of the natural and sole commerical source of these drugs.

A practical total synthesis of (±)-dihydrothebainone, (±)-nordihydrocodeinone and (±)-dihydrocodeinone in 37, 30 and 29% overall yield, respectively, from 3-methoxyphenethylamine has now been developed utilizing unprotected phenolic intermediates. Optical resolution of a key intermediate was accomplished in this study and application of the reaction sequence to the R-enantiomer will provide the (-)-isomers of dihydrothebainone, dihydrocodeinone and nordihydrocodeinone. The results described above when viewed in light of the work of others, indicated that practical total syntheses now appear available for a number of medically valuable opium derivatives, including the thebaine based drugs.

Publications:

Rice, Kenner C.: Synthetic Opium Alkaloids and Derivatives. A Short Total Synthesis of (±)-Dihydrothebainone, (±)-Dihydrocodeinone, and (±)-Nordihydrocodeinone as an Approach to a Practical Synthesis of Morphine, Codeine and Congeners, J. Org. Chem., in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19401-15 LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Natural Products as Probes for Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bernhard Witkop Chief, LC NIAMDD

COOPERATING UNITS (if any) O. Yonemitsu, Y. Kanaoka & T. Iwakuma, Univ. of Hokkaido, Sapporo, Japan; Masako Nakagawa, Faculty of Pharmaceutical Sciences, Chiba Univ. Japan; I. L. Karle, U.S. Naval Research Lab., Wash. D.C., Prof. E.X. Albuquerque, Univ. of Md. Med. School, E. Gössinger, Univ. of Vienna

LAB/BRANCH

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SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Histrionicotoxin and its congeners has become such an important probe for the study of ion (potassium) movement in (nicotinic) acetylcholine receptors that the demand for this compound exceeded supply. In order to close this gap a substitute was sought and found in the form of phencyclidine. 2. A novel powerful cholinergic agonist has been discovered in the toxic principle of the cyanophyte Anabaena flos-aquae, viz., the rigid bicyclic homologous tropane aminoketone anatoxin-a. 3. A synthesis of the toxin of the lady bug, adaline has been worked out by intramolecular 1,3-dipolar addition of a suitably substituted piperidine nitrone.

PHS-6040
(Rev. 10-76)

Project No. Z01 AM 19401-15

Project Description:

Objectives: To find known or new natural toxins with selective action on ion transport in muscarinic and nicotinic acetylcholine receptors or on transport of sodium, potassium or calcium in voltage-sensitive channels of electrogenic membranes in nerve and muscle.

Methods Employed: Ultraviolet irradiation, flash photolysis thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: Readily available synthetic phencyclidine is an excellent substitute for the histrionicotoxins in selectively blocking potassium transport in acetylcholine receptors. Anatoxin-a is the most powerful cholinergic agonist so far known. Dihydroadaline, the toxin of the lady bug competes with muscarinic but not with nicotinic acetylcholine receptors.

Significance to Biomedical Research: Agonists are involved in the gain or loss of receptors, i.e., synaptic plasticity. Such action has clinical implications for learning, memory and sleep patterns.

Proposed Course: Discovery and synthesis of further toxins as tools in neurobiology.

Honors:

1. Plenary Lecturer, International Congress: "Natural Products as Medicinal Agents", Strasbourg, France, July 6-11, 1980.
2. Member, Paul Ehrlich Foundation, Frankfurt for Five-year term, 1980-1985; appointment through President, Federal Republic of Germany.
3. Invited Lecturer, Nobel Jubilee Symposium, August 1981, Stockholm.

Publications:

E. X. Albuquerque, M.-C. Tsai, R. S. Aronstam, B. Witkop, A. T. Eldefrawi and M. E. Eldefrawi, Phencyclidine interactions with the ionic channel of the acetylcholine receptor and electrogenic membrane, Proc. Nat. Acad. Sci. U.S.A. 77, 1224-1228 (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19402-07 LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul F. Torrence	Research Chemist	NIAMDD/LC
Other:	Margaret I. Johnston	Staff Fellow	NIAMDD/LC
	Jiro Imai	Visiting Fellow	NIAMDD/LC

COOPERATING UNITS (if any) R.M. Friedman, LEP/NIAMDD; D.A. Epstein, LEP/NIAMDD; H. Jacobsen, LEP/NIAMDD; K. Zoon, LCB/NIAMDD; P. Bridgen, LCB/NIAMDD; S. Berger, LPP/NCI; E. DeClercq, Univ. of Leuven, Belgium; M. Mertes, Univ. Kansas, Lawrence, Kansas; G.-F. Huang, Tokyo Biochem. Res. Inst., Japan

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Metabolites

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Interferon-induced enzyme activities such as the oligo(2'→5') adenylate synthetase, the 67K dalton protein kinase and oligo(2'→5') A phosphordiesterase are investigated with a goal of understanding their role in the action of interferon, the induction of interferon by double-stranded RNA and, perhaps, control of cell growth and differentiation. An efficient high yield chemical synthesis of adenyl(2'→5')adenylyl(2'→5')adenosine (2',5'-oligo A core) has been completed to give material identical with that isolated from biological sources. High levels of oligo(2'→5')adenylate synthetase have been found in human lymphoblastoid cell lines such as Namalwa and 1788.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The structural, conformational and biochemical bases that account for the observed stringent structural requirements for nucleic acid interferon induction is sought. Interferon-induced enzymes, especially those apparently dependent on nucleic acids for activation, are studied in order to understand them as nucleic acid receptor systems as well as to elucidate their fundamental cellular role. Particular emphasis is currently placed on understanding the phenomenon of the dsRNA-mediated inhibition of protein synthesis in interferon-treated cells. The role of structure in determining the biological activity of (2'-5') oligo A is also under investigation.

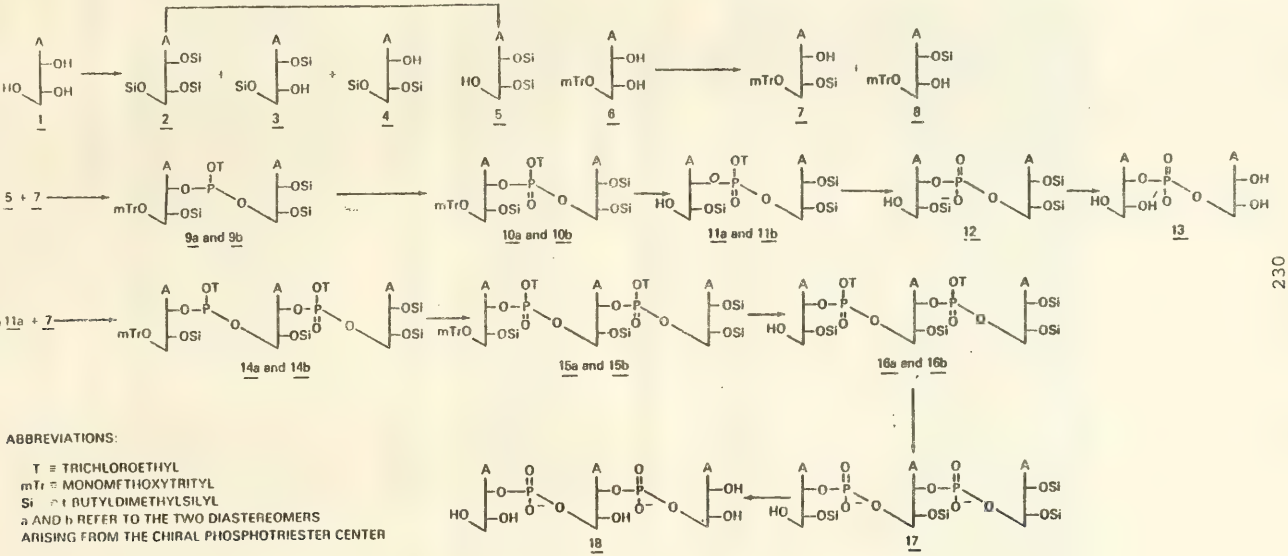
The antiviral and antimetabolic properties of new nucleoside analogs is also under investigation, on a collaborative basis.

Methods Employed: Thin-layer, paper, ion-exchange, gel and other types of column chromatography, liquid scintillation counting, ultracentrifugation, ultraviolet, visible, infrared and nuclear magnetic resonance, spectroscopy, mass spectroscopy, circular dichroism, tissue culture, gel electrophoresis, high performance liquid chromatography.

Major Findings:

2'5'-Oligo A Synthetase in Human Lymphoblastoid Cell Lines (Johnston, Zoon, Friedman and Torrence).

Namalwa cells originate from a Burkitt's lymphoma and have been transformed by Epstein-Barr Virus although they contain only a few incomplete copies of the virus in their genome and do not shed the virus. We have found that Namalwa cells have levels of 2'5'-oligo-A synthetase activity that equals or exceeds the levels associated with interferon-treated mouse L cells. Newcastle-Disease Virus infection of Namalwa cells enhance the level of synthetase by 2-4-fold while interferon is produced. Treatment of Namalwa cells with exogeneous interferon also slightly enhances synthetase activity levels. Namalwa cells are constitutive producers of interferon (5-15 units/ml), so that the possibility exists that they may activate themselves by constitutive production of interferon. When Namalwa cells are grown for three to four generations on the presence of excess antibody to F and Le interferon, synthetase activity drops three to ten-fold compared to untreated cells. This decrease in activity can be reversed by NDV infection to produce sufficient interferon to overwhelm the antisera absorption capacity. The synthetase activity that persists in the presence of excess antibody is still quite substantial and remains in the range of levels associated with some interferon-treated cells. It is established that interferon must be



ABBREVIATIONS:

T = TRICHLOROETHYL
mTr = MONOMETHOXYTRITYL
Si = t BUTYLDIMETHYLSILYL
a AND b REFER TO THE TWO DIASTEREOMERS ARISING FROM THE CHIRAL PHOSPHOTRIESTER CENTER

SYNTHESIS OF ADENYLYL (2'→5')-ADENYLYL(2'→5')-ADENOSINE

externalized to induce an antiviral state. Must it also be externalized to induce 2'5'-Oligo A synthetase? Anti-interferon serum decreases synthetase activity levels to those found in 1788 cells, a lymphoblastoid line which does not produce interferon constitutively. Since these 1788 cells have levels comparable to an interferon-treated mouse L cell, we are investigating whether or not lymphocytes themselves may have such elevated 2'5'-Oligo A synthetase levels insofar as EB virus trnasformation of B lymphocytes gives rises to the above lymphoblastoid lines (Johnston, Torrence, Friedman and Berger).

Chemical Synthesis of Adenylyl(2'→5')Adenylyl(2'→5')Adenosine (Imai and Torrence).

A complete and unequivocal chemical synthesis of the "core" of 2',5'-Oligo A has been completed in an overall yield (from 5 and 1) of > 40%.

This synthetic material showed the same proton and phosphorus nmr as "core" prepared in cell-free extracts of interferon-treated cells. It also comigrates with radiolabeled biosynthetic "core" on HPLC and a DEAE-cellulose column (linear salt gradient).

A similar synthesis has been reported by Olgivie and Theriault [Tetrahedron Lett. 2111 (1979)].

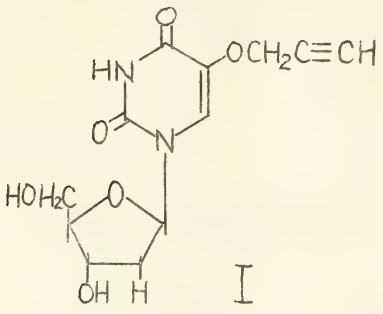
This synthesis not only verified the prepared structure of 2'5'-Oligo A, but provides a feasible synthetic route to analogs of 2'5'-Oligo A.

Nucleic Acid Structural Requirements for Activation of 2',5'-Oligo-adenylate Synthetase (Torrence, Johnston and Friedman).

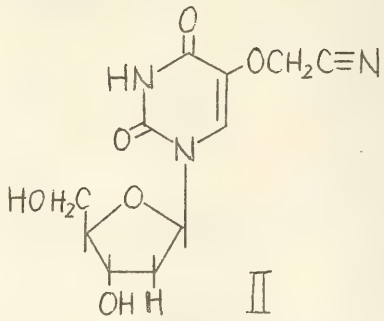
Using an assay (previously developed in this lab) that involves adsorption of 2',5'-oligo A synthetase to 2'5'-ADP-sepharose, we have examined the polynucleotide structural requirements for activation of 2',5'-oligo A synthetase. All single-stranded, triple-stranded and double-helical polymers substituted at the ribose C2' position were unable to activate the enzyme. Other polymers varied over 1000-fold in their ability to activate the enzyme. Poly(I)·poly(s^2C), poly(A)·poly(U) and duplexes based on poly(c^7A) were weak activators. In order of decreasing potencies, the strongest activators were P. chrysogemum ds RNA ≈ poly(I)·poly(C) > poly(c^7I)·poly(C) > poly(I)·poly(br^5C) ≈ poly(c^7I)·poly(br^5C) > bacteriophage QB RNA. These data suggest that the enzyme is quite sensitive to small changes in nucleic acid structure.

Synthesis and Antiherpes activity of 5-cyanomethyleneoxy-2'-deoxyuridine (Huang, Okada, De Clercq and Torrence).

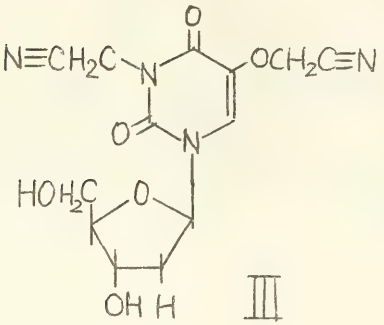
To study the influence of substitution of CN for C≡CH in the antiherpes virus nucleoside 5-propynyloxy-2'-deoxyuridine (I), 5-cyanomethyleneoxy-2'-doexyuridine (II) was prepared. When the potassium salt of 5-hydroxy-2'-deoxyuridine was reacted with iodoacetonitrile in dry DMF, the bisalkylated product, 3-cyanomethyl-5'-cyanomethyleneoxy-2'-deoxyuridine (III) was the major product with a lesser amount of 3-cyanomethyl-2'-deoxyuridine (IV) and only a trace of the desired product (II). To the contrary, when 5-hydroxy-2'-deoxyuridine was alkylated in water in the presence of one equivalent of KOH, compound II was the major product. Compound II showed an antiherpes virus activity comparable to I and araA in cultures of primary rabbit kidney (PRK) cells. Compound II did not inhibit incorporation of [Me-^{3}H]-dThd or [1',2'-^{3}H]dUrd into DNA of PRK cells; however, its antiherpes virus activity was completely reversed by either dThd or dUrd.



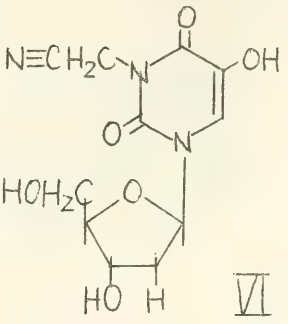
I



II



III



VI

These results suggest that substantial variation of the lipophilicity of the R group of the OCH_2R side chain at pyrimidine C-5 has little effect on the anti-herpes activity of such thymidine analogs.

Significance to Biomedical Research and the Program of the Institute: Studies such as those described herein showed lead to a better understanding of the mechanism of interferon action. They may also help unravel certain cellular control mechanisms that may be important not only as an antiviral defense but perhaps also in the control of growth, differentiation and development and thus perhaps neoplasia. Recently, it has been found that interferon is spontaneously produced during the course of certain auto-immune diseases such as systemic lupus erythematosus, rheumatoid arthritis, scleroderma and Sjögren's syndrome [Hooks et al., New Engl. J. Med. 301, 5-8 (1979)]. The role, if any, of interferon-induced enzymes on the above diseases is of obvious interest.

Proposed Course: The following topics are under active investigation:

1. The determination of the relationship of 2',5'-oligo A structure to protein synthesis inhibitory capacity. This will be achieved by the synthesis of various base and sugar modified analogs of 2',5'-oligo A. This study may enable us to find inhibitors of the action of 2',5'-oligo A, extend the biological helf-life and cell-penetration ability of 2',5'-oligo A and eventually to attempt to explore the 2',5'-oligo A system in a chemo-therapeutic sense.

2. Development of a radioimmunoassay for 2',5'-oligo A. Of the two methods currently available for assay of 2',5'-oligo A, one is of limited sensitivity and the other is extremely time-consuming. Thus a much faster sensitive assay will permit experiments on the level of 2',5'-oligo A in intact cells under various conditions.

3. Development of a speedy and reliable assay for the 2',5'-oligo A phosphodiesterase. A convenient and reliable assay for this enzyme has not yet been described. Since this enzyme represents a potential mechanism for cotrol of 2',5'-oligo A levels in cells, a reliable method for its estimation would be invaluable.

4. Nucleic acid structural requirements for activation of the 2',5'-oligo A synthetase and the 67K dalton protein kinase are being investigated in order to determine if there might be any relationship between these enzymes and interferon induction.

5. The possible biological roles of 2'5'-oligo A synthetase and 2',5'-oligo A itself are being investigated in human leukocytes and lymphoblastoid cell lines.

Publications:

Torrence, P. F., De Clercq, E., Descamps, J., Huang, G.-F. and Witkop, B. "Synthesis and Antiviral Activities of New 5-Substituted Pyrimidine Nucleoside Analogs", in Frontiers in Bioorganic Chemistry and Molecular Biology, Elsevier/North Holland Biomedical Press, eds. Y. A. Ovchinnikov and M. N. Kolosov, 59-85 (1979).

Chang, T.-C., Edwards, M. W., Torrence, P. F. and Mertes, M. P. "5-Cyano-2'-deoxyuridine-5'-phosphate: A Potent competitive inhibitor of thymidylate synthetase", J. Med. Chem. 22, 1137-1139 (1979).

De Clercq, E., Descamps, J., Verhelst, G., Walker, R. T., Jones, A. S., Torrence, P. F. and Shugar, D. "Comparative efficacy of different drugs against different herpes simplex virus strains", J. Infect. Dis. 141, 563-574 (1980).

Descamps, J., De Clercq, E., Barr, P. J., Jones, A. S., Walker, R. T., Torrence, P. F. and Shugar, D. "Relative potencies of different anti-herpes agents in the topical treatment of cutaneous herpes simplex virus infection of athymic nude mice", Antimicrob. Ag. Chemother. 16, 680-682 (1979).

De Clercq, E., Huang, G.-F., Bhooshan, B., Ledley, G. and Torrence, P. F. "Interferon induction by mismatched analogs of polyinosinic acid·polycytidylic acid[$(I_x,U)_n \cdot (C)_n$]", Nucleic Acids Research 7, 2003-2014 (1980).

Epstein, D. A., Torrence, P. F. and Friedman, R. M. "Inhibition by double-stranded RNA of a phosphoprotein phosphatase present in interferon-treated cells", Proc. Nat. Acad. Sci. U.S.A. 77, 107-111 (1980).

Torrence, P. F. "Synthetic inducers of interferons", in Interferon, ed. L. A. Kohn, Marcel-Dekker, N. Y., N. Y., in press.

Epstein, D. A., Torrence, P. F. and Friedman, R. M. "$(I)_n \cdot (C)_n$ Inhibition of phosphoprotein phosphatase activity in extracts of interferon-treated cells", in "The Interferons", a volume of Methods in Enzymology, ed. S. Pesta, Academic Press, N. Y., N. Y., in press.

Johnston, M. I., Friedman, R. M. and Torrence, P. F. "Assay of (2'-5')Oligo A Synthetase with 2',5' ADP-Sepharose" in Ibid., in press.

Torrence, P. F. and De Clercq, E. "Interferon inducers, general survey and classification", in Ibid., in press.

Torrence, P. F. "Preparation of a synthetic polynucleotide interferon inducer", Ibid., in press.

Imai, J. and Torrence, P. F. "An efficient chemical synthesis of adenylyl (2'→5')adenylyl(2'→5')adenosine", Ibid., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19408-07 LC

PERIOD COVERED October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Central Nervous System Agents Based on the Piperidine Ring System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James A. Waters	Research Chemist	NIAMDD/LC
Other:	E.X. Albuquerque	Professor	U. of MD, Baltimore
	C.E. Bolcik	Lab Aide (SIS)	NIAMDD/LC
	E. Brochmann-Hanssen	Professor	U. of Calif., S.F.
	J.W. Daly	Lab. Chief	NIAMDD/LBC
	A.E. Jacobson	Research Chemist	NIAMDD/LC
	E.L. May	Adjunct Professor	Med. Coll. Va. (Richmond, Va.)
	B. Witkop	Lab. Chief	NIAMDD/LC

COOPERATING UNITS (if any)

Antiepileptic Drug Development Program, Epilepsy Branch, NINCDS, NIH

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Metabolites

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Aromatic, heterocyclic and cycloalkyl carboxylates of the 4-piperidinol system and related tertiary aminoalcohols have been synthesized for pharmacological and biochemical study. Two CNS classes have been found: (1) non-addicting analgesics (mice, Rhesus monkeys); (2) antiepileptics (mice). A tritiated piperidinol ester has been prepared for non-opiate receptor study. Certain compounds have been selected for the study of their effect on the Na^+/K^+ channels of electrogenic membranes. Quantitative and qualitative structure-activity relationships have been made.

PHS-6040
(Rev. 10-76)

Project No. Z01 AM 19408-07

Project Description:

Objectives: To study the biochemical and pharmacological actions of new synthetic antiepileptics and non-addicting analgesics.

Methods Employed: Aromatic, heterocyclic and cycloalkyl carboxylates of the 4-piperidinol system and related tertiary aminoalcohols and aralkylpiperidines have been synthesized and assayed for antiepileptic activity (maximal electroshock and subcutaneous pentylenetetrazole seizure, mice), analgesic activity (hot-plate, Nilsen, tail-flick and PPQ abdominal stretch, mice), physical dependence liability (single dose suppression, monkeys) and abuse liability (self-administration, monkeys).

Major Findings: Sixteen esters of 1-methyl-4-piperidinol, from a group of one hundred and twenty-six synthetic compounds, exhibited analgesic activity in the codeine-morphine range (hot-plate). In general, these non-quaternary C-4 piperidinol esters showed no physical dependence of the morphine-type. Self-injection behavioral studies indicated very low or no abuse potential (monkeys). Sixty-six compounds were assayed for antiepileptic activity (MES). Those substituted at the 2 position of the aryl ring with phenyl, benzyl or phenethyl exhibited antiepileptic activity in the phenobarbital-methsuximide potency range. This is a relatively strict structural requirement for enhancing antiepileptic activity in these piperidinol esters.

Significance to Biomedical Research: New analgesics which lack physical dependence, abuse liability and other side-effects of the morphine-type narcotics are needed for clinical use and for use in addiction programs. The recent finding of long-term toxic effects of some of the standard antiepileptics (i.e., Dilantin) increases the need for drugs with greater CNS selectivity. The study of these substrates at specific receptor sites and their effect on ion transport will provide a better understanding of their biochemical mechanisms.

Proposed Course: A tritiated substrate in the analgesic series will be used to study agonist-agonist interactions in an effort to identify a non-opiate type receptor. Studies will also be made on selected piperidinol esters to determine their effect on the Na^{+}/K^{+} channels of electrogenic membranes. NINCDS is examining several compounds in their advanced antiepileptic assays.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19410-02 LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Synthesis of Gephyrotoxin 223 Stereoisomers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Thomas F. Spande Research Chemist NIAMDD/LC

COOPERATING UNITS (if any)

John W. Daly, Chief, Laboratory of Bioorganic Chemistry, NIAMDD

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Metabolites

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new indolizine synthesis has been devised which exploits novel acyl alkoxycyclopropane chemistry. Catalytic or chemical reductions should produce Gephyrotoxin 223 stereoisomers for comparison with a natural indolizidine isolated from the skins of the Colombian frog Dendrobates histrionicus.

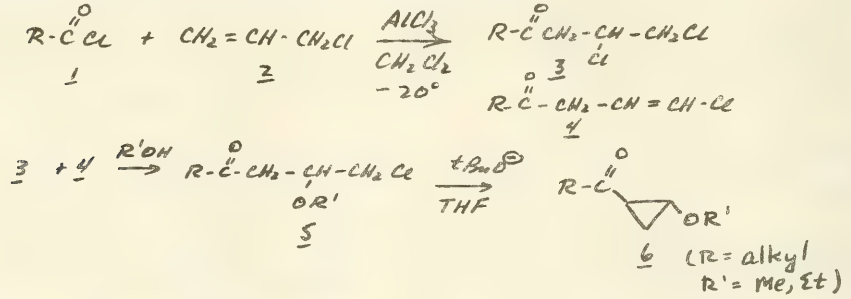
PHS-6040
(Rev. 10-76)

Project No. Z01 AM 19410-02

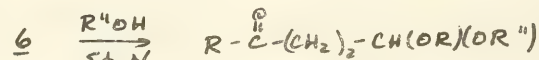
Project Description:

Objectives: Insufficient material is available from the natural source for biological testing and a thorough structure proof necessitating a practical synthesis.

Major Findings: The aryl alkoxy cyclopropane intermediate 6 are easily obtained by the following general scheme.

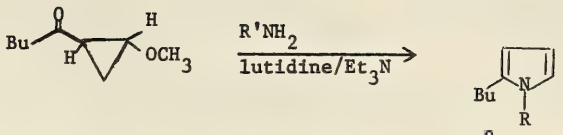


PMR indicates the configuration of 6 to be solely trans. Compound 6 reacts exothermically with alcohols to form 4-keto acetals in excellent yield. Mixed acetals are formed when $R^{I} \neq R^{II}$ and a trace of triethylamine



is present. Otherwise equilibration occurs and a mixture of the three possible acetals results.

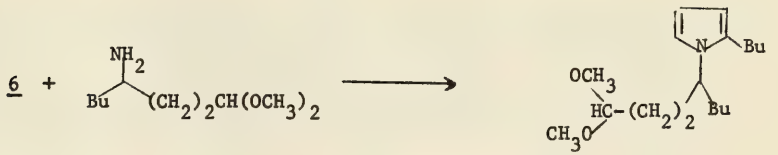
Such 4-keto acetals, important precursors of 2-alkyl-pyrroles and -furans, are otherwise quite difficult to obtain directly. Such keto aldehyde equivalents are also useful in synthesizing jasmone and prostaglandin intermediates. With acetic or formic acids, exothermic reaction of 6 affords 4-keto α-alkoxy esters, $RCO(CH_2)_2CH(OR')(O_2CR'')$; R''=H,$CH_3$ Most importantly, when 6 are refluxed in acetonitrile on toluene containing a primary amine, lutidine and a catalytic amount of trifluoroacetic acid. 1,2-disubstituted pyrroles (8) result in good yield. This represents a convenient new pyrrole synthesis.



6 8

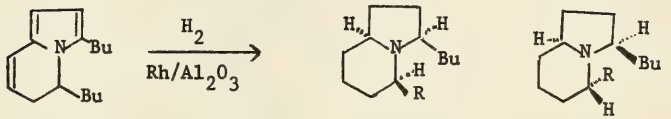
(R = Ø CH_2, n-Bu, i-Bu, s-Bu, t-Bu, p-anisyl, etc.)

Employing this reaction with the γ-amino acetal 9 prepared from 7 (R=Bu, R'-R''=me) by axime formation and reduction, the pyrrole 10 results.



9 10

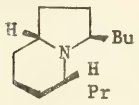
This is cyclized with TFA, $ZnCl_2$ or $BF_3 \cdot Et_2O$ to the dihydroindolizine 11. It is anticipated that catalytic reduction of 11 should afford the following two Gephyrotoxin 223 homologs (12a, 12b).



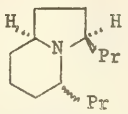
11

12a R=Bu (major) 12b R=Bu (minor)
13a R=Pr 13b R=Pr

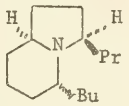
Proposed Course: After optimizing the cyclization and reduction conditions, I shall prepare 13a and 13b by reaction of Pr-CH(NH_2)-$CH_2CH_2CH(OCH_3)_2$ with 6 (R=Bu, R'=CH_3). These will be compared with the natural material. It is fairly likely that one of these will be identical with the natural material since the stereoisomer 13c recently synthesized by MacDonald was shown not to be the natural isomer.



13c



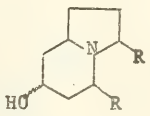
13d



13e

Separated Gephyrotoxin 223 stereoisomers will be compared with the natural material by GC-MS. Should one prove identical, a scaled up synthesis will be undertaken and various homologs and analogs will be prepared for biological testing (with John Daly). Having 6 (R=Pr, R'=me) in hand as an intermediate for 9 (R=Pr, R'=me), should make possible an easy synthesis of the nor. tmxin 13d. Likewise the structure 13e with interchanged side chains should result from 6 (R=Pr, R'=me) and 9 (R=Bu, R'=me).

The dihydroindolizines 11 should provide a useful entry into piperidine-ring hydroxylated materials, e.g. 14, which being 3-hydroxy amines might be anticipated to have significant neurophysiological activity (cf. histrionicotoxins).



14

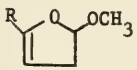
Hydroboration with hindered boranes followed by alkaline hydrolysis and reduction will be tried as a route to 14.

Gephyrotoxins labeled with D or T in the side chain attached to the piperidine ring should be possible using labeled amino acetals (e.g. R-CD_2-$CH(NH_2)CH_2CH_2(OCH_3)_2$ or $RCH_2CD(NH_2)CH_1CH_2(OCH_3)_2$). The former amino acetal could be prepared easily by alkoxide catalyzed deuteration or tritiation of 6 since only the non-cyclopropane-ring positions adjacent to the carbamyl group

are observed to exchange. The latter amino acetal should result with catalytic reduction of the oxime with deuterium gas. Deuterated or tritiated 6 should place the label in the side chain attached to the pyrrolidine ring.

A pyrrolizidine synthesis (cf. Senecio alkaloids) will be attempted using 6 with amino acetals ($R\text{-}CH(NH_2)\text{-}CH_2\text{-}CH(OR')_2$). A recently isolated insect phenomenon is such a 3,5-dialkyl pyrrolizidine.

Finally, the mechanism for the interesting pyrrole conversion from 6 will be studied. In the toluene system, at least, it is likely that the dihydrofuran 15 is an intermediate. This can be prepared easily from 6 (R'=me) in DMSO with $PyH^{\oplus}$ $OTs^{\ominus}$.



15

Publications:

Thomas F. Spande, The Syntheses of Two Novel Phosphorylcholine Esters for Use in Immunological Studies. J. Org. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19600-15 LC

PERIOD COVERED

October 1, 1979, through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Oxidation Mechanisms in Metabolic Processes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Louis A Cohen Chief, Section on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been temporarily discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19601-13 LC

PERIOD COVERED

October 1, 1979, through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chemical Modification and Cleavage of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Louis A. Cohen Chief, Section on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been temporarily discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19602-14 LC

PERIOD COVERED

October 1, 1979, through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cleavage of Peptide Bonds by Intramolecular Participation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Louis A. Cohen Chief, Section on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

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Laboratory of Chemistry

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Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been temporarily discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19603-04 LC

PERIOD COVERED

October 1, 1979, through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Fluoro Analogs of Enzyme Substrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Louis A. Cohen	Chief, Section on Biochemical Mechanisms	LC NIAMDD
OTHER:	Kenneth L. Kirk	Research Chemist	LC NIAMDD
	Olarongbe Olubajo	Visiting Fellow (Arr. March 1979)	LC NIAMDD
	Shuji Kondo	Visiting Fellow (Term. April 1980)	LC NIAMDD
	Chaim Rav-Acha	Visiting Fellow (Term. August 1980)	LC NIAMDD

COOPERATING UNITS (if any)

P. F. Torrence, LC NIAMDD
C. R. Creveling, LBC NIAMDD

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Ring-fluorinated imidazoles show widespread and significant activity as enzyme substrates. 2-Fluorohistidine, but not the 4-fluoro isomer, is rapidly incorporated into newly synthesized animal or bacterial protein, in place of histidine. The resulting enzymes may be structurally intact, but some cannot function catalytically because the pK of the imidazole ring has been greatly depressed. 2-Fluorohistidine also serves to produce leucopenia, temporary regression of the mouse spleen, thymus, lymph nodes, and hypocellularity of the bone marrow. In mice infected with leukemia virus P-388, this analog prevents the normally massive increase in leukocyte levels and increases survival time up to 50%. Ultimate death may be due to causes other than leukemia. This anti-leukemic action may be unrelated to production of false protein, and may result from a block in the utilization of histidine as a one-carbon donor in purine biosynthesis. The block would result from the powerful inhibition of urocanase by 2-fluorocanic acid, a metabolite of 2-fluorohistidine.

PHS-6040
(Rev. 10-76)

Project No. Z01 AM 19603-04 LC

Project Description:

Investigation of the biochemical and pharmacological properties of ring-fluorinated imidazoles, as tools for the study of enzyme structure and function and as potential therapeutic agents.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole had yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds and to test their abilities to serve as enzyme substrates or inhibitors and as replacement for histidine in proteins or for purines in DNA and RNA. To evaluate the fluoroimidazoles as antileukemic, antiviral, and immunosuppressive agents.

Methods Employed: Various enzyme assays, enzyme fractionation and purification methods are employed. Automatic cell counters to determine leukocyte levels. Standard techniques for manipulation of laboratory mice. Culture of yeasts and E. Coli.

Major Findings: Studies have continued on the incorporation of 2-fluorohistidine into various protein-synthesizing systems, from microorganisms to the whole mouse. Earlier experiments had involved 2-F-his with tritium labeling in the imidazole ring. The stability of the label to exchange is not quite as high as desirable, and efforts were made to prepare the same compound with a radioactive label on the amino acid side chain. For this purpose, the corresponding malonic acid derivative has been synthesized; decarboxylation in deuterium oxide provides an amino acid with label at the α-position. Hence, the tritiated material will be obtainable by an analogous procedure.

Studies on the antibacterial activity of 2-fluorohistidine have shown that the cessation of growth of E. coli is due, at least in part, to a powerful inhibition of the first enzyme in the histidine biosynthetic pathway, phosphoribose-ATP pyrophosphorylase. Further studies have now shown that the inhibition can be overcome by prolonged incubation, and that the resistant strain (presumably a mutant) is no longer sensitive to 2-fluorohistidine. Since we have demonstrated that the analog is partially incorporated into protein (in place of histidine) during the brief growth cycle, the mutant may be able to incorporate much larger amounts, thus permitting the biosynthesis and isolation of various pseudoenzymes containing fluorine. These analogs are expected to be useful for study of enzyme mechanism by fluorine NMR and by other techniques.

Significance to Bio-medical Research and the Program of the Institute: Studies to date indicate fluoroimidazoles to have a variety of properties in biological systems: enzyme inhibitors, histamine agonists, analogs of

histidine, antibacterial, antiviral, and antileukemic agents. These compounds have already proved their usefulness in studies of enzyme structure and function, as inhibitors of purine biosynthesis, involvement in cell division, etc. The possibility of therapeutic use for one or more of these compounds should also be considered.

Proposed Course: Additional fluoro analogs are being synthesized for biological evaluation. More detailed studies with enzymes are in progress, as well as efforts to isolate fluorine-containing proteins. The mechanism of the antileukemic effect is being investigated in detail.

Publications:

P. F. Torrence, R. M. Friedman, K. L. Kirk, L. A. Cohen, and C. R. Creveling: 2-Fluorohistidine: Effects on Protein Synthesis in Cell-Free Systems in Mouse-L-Cells. Biochem. Pharmacol. 28, 1565-1567, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19604-10 LC

PERIOD COVERED: October 1, 1979, through September 30, 1980

TITLE OF PROJECT (80 characters or less)

General Principles of Enzyme Catalysis and Simulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Louis A. Cohen	Chief, Section on Biochemical Mechanisms	LC NIAMDD
OTHER:	Michael King	Guest Worker (Research Chemist - Summer Only)	GWU
	Michael Channing	Staff Fellow (Arr. April 1979)	

COOPERATING UNITS (if any): None

LAB/BRANCH: Laboratory of Chemistry

SECTION: Section on Biochemical Mechanisms

INSTITUTE AND LOCATION: NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.4	.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have advanced the theory that a major portion of enzymatic catalysis is achieved by activation of the substrate during binding. To support the theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen in a single, favorable conformation and by having the interacting functions brought into the closest possible juxtaposition. (stereopopulation control). These compounds undergo intramolecular reactions at rates comparable to those catalyzed by enzymes, sometimes even too fast to measure. It has been possible to generate high energy bonds within these models without energy input, simply using the free energy of formation of water and conformational restriction as the driving forces. In current work, models for sulfhydryl enzymes, oxidative phosphorylation and ion pumps are being developed. Techniques are being developed to elucidate molecular conformation in solution, based on ^{13}C and ^{19}F nmr spectroscopy, as well as computer calculations of conformational energies (CAMSEQ).

PHS-6040
(Rev. 10-76)

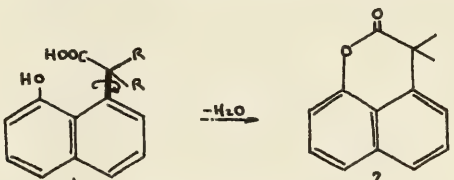
Project Description:

A study of the principles used by enzymes to accelerate chemical reactions. Model compounds are designed and synthesized which are capable of undergoing various reactions at rates comparable to those of enzymes and under the same reaction conditions. Recognition of the principal devices used by enzymes will permit the design of more effective drugs and enzyme inhibitors or substrates.

Objectives: The rates of enzyme-catalyzed reactions exceed those of simple test-tube analogs by factors of 10^{10} - 10^{20}. In order to account for such a phenomenal effect, model compounds which duplicate one or more of an enzyme's special powers are studied to learn more about the vastly complex protein catalyst. In the belief that the principal enzymatic device is stereopopulation control (near perfect orientation of functional groups), model compounds have been designed and synthesized in which rotation of covalent bonds has been severely restricted. By bringing two functional groups into very close proximity and orientation, rates of reaction comparable to those of enzymes can be achieved in the test tube.

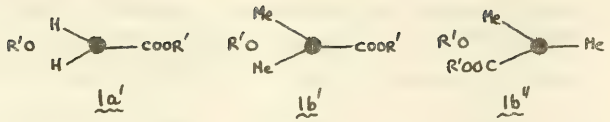
Methods Employed: Spectroscopic methods are used to elucidate the structures of synthetic products and to follow rates of reaction. Various chromatographic procedures are employed for analysis and purification. ^{1}H, ^{13}C and ^{19}F NMR spectra are recorded over the temperature range -100 to +180°C, to detect conformational changes in solution. X-ray crystallography is used to determine conformation in the solid state. Free energies of the various conformers are calculated by use of computer programs (CAMSEQ).

Major Findings: In continuation of our efforts to understand the role of conformational restriction in intramolecular rate enhancement, we have developed synthetic methods for compounds 1a and 1b, and have studied the kinetics of conversion of these compounds to their lactones, 2. The peri-naphthalene system was



1 a, R=H; b, R=CH3 2

chosen because of its simplicity, i.e., only one bond (heavy line) can rotate to produce conformational isomers. Compound 1b was found to cyclize at least 10^5 as rapidly as 1a and, even at pH 11, cyclization of 1b is complete within a few seconds. We consider that, in 1a, the preferred rotamer has the carboxyl group positioned as far from the phenol as possible, in order to minimize dipolar repulsion and steric crowding between the bulky solvated dipoles (1a').



1a' 1b' 1b''

In the analogous rotamer for 1b, however, the solvated phenol would have to be flanked by two bulky methyl groups (1b') or by one methyl and a carboxyl (1b''). Since methyl is is much larger than carboxyl, the preferred rotamer should now be 1b'' and, thus, ring closure would be faster because the latter conformation is much closer to the geometry of the transition state. At ambient temperature the NMR signal for the methyl groups of 1b (R = alkyl) appears as a sharp singlet. As the temperature is reduced, the signal broadens and, at -20°, has separated into two distinct singlets of equal area. Since the methyl groups in 1b'' are chemically nonidentical, the low temperature spectrum must represent 1b', supporting the argument based on kinetic data.

Significance to Biomedical Research and the Program of the Institute: Despite the vast amount of research invested in the elucidation of the pathways of specific enzyme-catalyzed reactions, justifications of the rates of these reactions, in comparison with their nonenzymatic counterparts, are still in a primitive state. A sound understanding of the means by which enzymes operate so effectively would lead not only to a better appreciation of biochemical processes in general, but also to a more rational approach to drug design.

Proposed Course: Utilizing the principles of stereopopulation control already developed, additional models will be studied to produce even faster reaction rates than those already observed. The principle will also be extended to other types of reactions, such as hydride transfer and addition/elimination. The control principle will also be used to design conformationally restricted analogs of various drugs with a view to enhancement of activity and reduction on side-effects.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19605-04

PERIOD COVERED

October 1, 1979, through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chemistry of Substituted Imidazoles

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Louis A. Cohen	Chief, Section on Biochemical Mechanisms	LC NIAMDD
OTHER:	Kenneth L. Kirk	Research Chemist	LC NIAMDD
	Robert Jerussi	Guest Wroker	LC NIAMDD
	Olarongbe Olubajo	Visiting Fellow (Arr. March 1979)	LC NIAMDD
	Shuji Kondo	Visiting Fellow (Term. April 1980)	LC NIAMDD
	Michael Channing	Staff Fellow (Arr. April 1979)	LC NIAMDD
	Chaim Rav-Acha	Visiting Fellow (Term. August 1980)	LC NIAMDD

COOPERATING UNITS (if any)

Hiroshi Kimoto, Industrial Research Institute, Nagoya, Japan

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	2.3	.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Discovery of the widespread and impressive properties of fluoroimidazoles as biological substrates and analogs has provided the impetus for the synthesis of other substituted imidazoles. Novel methods have been developed for the synthesis of previously unknown or difficultly accessible 2-substituted imidazoles. Thus, all 2-halohistamines and 2-halohistidines are now available as well as analogs containing the amino, formyl, cyano, and nitro and azido groups. Discovery of a method for the direct introduction of the 2-amino group (via addition of the imidazole carbanion to phenyl azide) provides a basis for the synthesis of the song sought after 2,4-difluoroimidazoles. 2-Trifluoromethylimidazoles, obtained by another novel route, are readily converted to 2-carboxy, 2-carbethoxy, and 2-cyanoimidazoles. The decarboxylation of 1-methyl-4, 5-dicarboxyimidazole occurs exclusively at C-4, showing the mechanism to involve an ylid rather than carbanion. pH dependence studies show that the decarboxylation of nitroimidazolecarboxylic acids also involves an imidazolium ion, despite the extremely low basicity of the ring.

Project No. Z01 AM 19605-04 LC

Project Description:

Development of new methods for the synthesis of fluoro and other analogs of biochemically significant compounds and examination of their properties as enzyme inhibitors and as potential drugs.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole had yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds and to test their abilities to serve as enzyme substrates or inhibitors and as replacement for histidine in proteins or for purines in DNA and RNA. Discovery of the widespread and significant biological properties of fluoroimidazoles provided the impetus for the synthesis of other substituted imidazoles.

Methods Employed: Ultraviolet irradiation is used as the key step in the introduction of fluorine. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure. Various chromatographic procedures are used for analysis and purification of materials.

Major Findings: 2-Trifluoromethyl-L-histidine and 2-trifluoromethylhistamine are easily obtained from L-histidine and histamine by a three-step synthesis (see earlier reports). These compounds have now been converted into new substrate analogs: 2-carboxy (by alkaline hydrolysis); 2-carbomethoxy (by methanolysis); and 2-cyano (by ammonolysis). The 2-cyano analog was found to decompose during chromatographic purification and it beccame necessary to devise an alternative synthesis. The α-amino groups of the 2-trifluoromethyl analogs were protected as their tert-butoxycarbonyl derivatives; these materials were then subjected to ammonolysis and the resulting 2-cyanoimidazoles were readily purified. Finally, the protecting groups were removed by hydrogen chloride in ethyl acetate to provide pure samples of the desired compounds. Thus a variety of new analogs of histidine and histamine have been made easily accessible for biochemical and pharmacological evaluation.

Trifluoromethylimidazoles show the unique property of losing hydrogen fluoride above pH 9 to form a metastable difluorodiazafulvene. Such intermediates, derived from histidine and histamine, may be able to serve as covalent affinity labels for enzyme and receptor binding sites. Since the loss of hydrogen fluoride is very slow at physiological pH, we have been exploring methods to increase reactivity by introduction of an additional substituent into the imidazole ring. Thus, 2-trifluoromethylhistidine was successfully chlorinated at C-4 and the reactivity of this derivative is being examined. In attempts to nitrate the histidine analog, a novel reaction was discovered whereby strong acid effects the replacement of the trifluoromethyl group by a fluorine atom.

Should this reaction prove to be practical on a large scale, it would provide a new and valuable route to 2-fluoroimidazoles, which compounds are obtainable only by photochemical procedures in modest yield. 4-Trifluoromethylimidazoles lose hydrogen fluoride ca. ten times as rapidly as the 2-isomers, and may therefore be of greater value as biological tools. Unfortunately, 4-trifluoromethylimidazoles cannot be obtained by the same direct methods as for the 2-isomers, and classical syntheses of the unknown 4-trifluoromethylhistidine and histamine would require at least ten steps each. We attempted, therefore, to develop nonclassical, direct routes to such compounds and have succeeded in this effort. When simple imidazoles are irradiated in the presence of trifluoromethyl iodide, the trifluoromethyl group becomes attached directly to the ring, mixtures of the 2- and 4-isomers being obtained in roughly equal amounts. This new synthetic method is now being attempted with histidine and histamine.

Our new synthetic method for 2-nitroimidazoles, involving nitration of the C-2 carbanion with alkyl nitrates, has been extended to the synthesis of 2-nitrohistidine. 2-Nitroimidazoles have found wide use as antibiotics and antiparasitic agents, and are currently undergoing extensive evaluation as sensitizers in radiation therapy and as cytotoxic agents highly selective for cancer cells. Thus, the new synthetic method will permit the synthesis and evaluation of a wide variety of new 2-nitroimidazoles. We have initiated an investigation into the molecular mechanism of the cytotoxic effect, which is thought to involve interaction with extracellular sulfhydryl groups. To date, we have demonstrated that the decomposition of 1-methyl-2-nitroimidazole by aqueous base is not initiated by displacement of the nitro group or by attack at C-2, but by conjugate addition to the 4,5-double bond of the ring; subsequently, the ring opens to give N-methylurea and glyoxalate. The nitro compound does not react with simple thiols under physiological conditions and the biological reaction would appear to be more complex than has been postulated.

Our discovery of the antileukemic activity of 2-fluorohistidine, coupled with the demonstration that 2-fluorourocanic acid is a powerful inhibitor of urocanase, have led us to speculate that the antileukemic effect may involve a block of the utilization of histidine as a one-carbon donor for purine biosynthesis or for cell division. We have been involved, therefore, in efforts to synthesize a number of analogs of urocanic acid for pharmacological evaluation. Although certain analogs can be obtained on a microscale by deamination of the corresponding histidine analogs with histidine ammonis-lyase, this method is impractical for large quantities. Synthetic methods, on the other hand, are complicated because of the reactive double bond in the side chain. We have now developed a procedure for reduction of urocanic acid to the primary allylic alcohol, urocanol. Following introduction of the desired 2-substituent, the alcohol moiety will be reoxidized to the carboxylic acid.

The first step in the synthesis of 2-trifluoromethylhistidine involves cleavage of the imidazole ring of histidine with benzoyl chloride and strong alkali (Bamberger cleavage). This procedure does not work with urocanic acid or with any imidazoale carrying an electronegative substituent. By elucidating the mechanism of the Bamberger cleavage, we were able to predict that certain

other acid chlorides should be effective for the less reactive imidazoles. Indeed, Bamberger cleavage of urocanic acid was achieved by use of carbobenzoxy chloride and the product has been converted into 2-trifluoromethylurocanic acid. The latter compound can now serve as a starting point for the synthesis of other 2-substituted urocanic acids.

Imidazoles exhibit a variety of anomalous physical and chemical properties which can best be explained by considering the ring to be only slightly aromatic, or nonaromatic, in character. We have sought to evaluate the magnitude of pi-electron transmission across the ring by studying the ability of a nitro group to activate the protons of a distal methyl group toward isotope exchange. While activation between positions 4 and 5 is very strong, that between 2 and 4 or 2 and 5 is weak, supporting the argument that an aromatic ring current, if present at all, is not significant. This work also demonstrated that introduction of the N-methyl group effects bond fixation in the amidine portion of the ring, and that the two nitrogen atoms are no longer equivalent in electron transmission.

Significance to Bio-medical Research and the Program of the Institute: Fluoroimidazoles have already been found to be powerful tools for the investigation of enzyme structure and function, as inhibitors of metabolic pathways and as potential therapeutic agents. We believe that parallel properties will be observed with other analogs of natural imidazoles, possibly with even greater potency. These analogs are designed to answer specific questions regarding the steric and geometric limitations of natural substrates, the importance of pK_1 and pK_2 of imidazoles to their biological behavior, and the role of ring current effects.

Proposed Course: Continued efforts will be made to develop synthetic routes to difficultly accessible imidazoles. Studies will be continued in an effort to rationalize the anomalous physical and chemical properties of the imidazole series. Many of the compounds will be evaluated for their biological properties.

Publications:

Kimoto, H., and Cohen, L. A.: Fluorine Reactivity in 2-Trifluoromethylimidazoles. J. Org. Chem. 44, 2902-2906 (1979).

Takeuchi, Y., Kirk, K. L., and Cohen, L. A.: Acid-Catalyzed Isotope Exchange of Ring Hydrogens in Fluoroimidazoles. J. Org. Chem. 44, 4240-4242 (1979).

Takeuchi, Y., Kirk, K. L., and Cohen, L. A.: Imidazole Cyclotrimers (Trimidizoles), a Novel Heteroannular Series. J. Org. Chem. 44, 4243-4246 (1979).

Kirk, K. L.: A Facile Synthesis of 2-Substituted Imidazoles. J. Org. Chem. 43, 4381-4383 (1978).

Kirk, K. L.: A Convenient Synthesis of 3-Fluoro-L-Tyrosine and 3,5-Difluoro-L-Tyrosine. J. Org. Chem. 45, 2015-2016 (1980).

Project No. Z01 AM 19605-04 LC

Cohen, L. A., Kirk, K. L., and Davis, D. P.: Synthesis of 2-Substituted Histidines. American Chemical Society, Abstracts 178th Meeting, Sept. 1979, ORGN 122.

Kimoto, H. and Cohen, L. A.: New Histamine and Histidine Analogs *via* Transformation of the 2-Trifluoromethyl Group. J. Org. Chem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19606-04 LC

PERIOD COVERED

October 1, 1979, through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Halogenated Biogenic Amines in Biochemistry and Pharmacology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth L. Kirk	Research Chemist	LC NIAMDD
OTHER:	Daniele Cantacuzene	Guest Worker	LC NIAMDD
	Olorongbe Olubajo	Visiting Fellow (Arr. March 1979)	LC NIAMDD
	Cyrus R. Creveling	Research Chemist	LBC NIAMDD
	John Daly	Chief, Laboratory of Bioorganic Chemistry	LBC NIAMDD

COOPERATING UNITS (if any) L. I. Goldberg, J. Kohli, U. of Chicago; C. Dobsen, F. M. Poulsen, Harvard U.; J. Martin, Washington U.; S. May, Georgia Tech.; D. Murphy, J. Costa, NIMH; D. C. Klein, D. Auerbach, NICHD

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.6	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fluorinated analogs of norepinephrine, isoproterenol, phenylephrine, tyramine, dopamine, melatonin, serotonin, DOPA, and α-methylDOPA have been synthesized. Biochemical and biophysical studies on these analogs have revealed important information concerning conformational requirements for norepinephrine in interaction with α and β-adrenergic receptors, and the role of phenolic pK and lipophilicity in determining activity of biogenic amines. The use of ^{19}F NMR spectroscopy as a probe for molecular motion has revealed important information concerning the mechanism of serotonin storage in human platelets.

PHS-6040
(Rev. 10-76)

Project Description:

Development of new methods for the synthesis of halogenated analogs of biogenic amines, study of their physical properties, and evaluation of their behavior in biological systems as antagonists or agonists.

Objectives: Halogenated analogs have proved to be powerful tools for the study of transport, storage, release, and action of biogenic amines, since they simulate the geometry of the natural compounds so well. Continued studies should provide significant information about the roles of these essential metabolic species and possibly lead to therepeutic use.

Methods Employed: Ultraviolet irradiation and various chemical methods are used for the introduction of halogen into biogenic amines. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure and purity. Various chromatographic procedures are used for purification and analysis of materials. Electron energy-loss spectroscopy is used to detect and localize fluorine-containing materials in tissues.

Major Findings: The dramatic differences in adrenergic agonist specificities previously described for the isomeric ring-fluorinated norepinephrines (FNE) have been studied in greater detail. To gain insight into the mechanisim(s) of these specificities, the binding of FNEs and fluorodopamines (FDA) to adrenergic and dopaminergic receptors in rat brain membranes has been studied in detail. In the NE series, binding to β_1-adrenergic receptors in rat cerebral cortical membranes follows the order: NE > 2FNE > 5FNE >> 6FNE. For β_2-adrenergic receptors, the order was: NE $\sim$ 2FNE $\sim$ 5FNE >> 6FNE. In contrast, for the α-adrenergic receptor, the order of potency was: 6FNE $\sim$ NE > 5FNE >> 2FNE. Thus, the binding studies are in agreement with the previously observed selectivities for the FNEs, i.e, 6FNE is a relatively selective α-adrenergic agonist while 2FNE is a relatively specific β-adrenergic agonist.

2,5,and 6-FDA are more potent ligands for the α_1-adrenergic receptors than for α_2 and β-receptors, but the specificity is independent of fluorine position with respect to the dopaminergic receptor. DA and the three FDAs are essentially equipotent. The lack of major differences in binding of the isomeric FDAs to adrenergic receptors suggests that the specificities shown by FNEs is due to interaction of the ethanolamine side chain with an ortho situated fluorine substituent, and not due to altered phenolic pKa.

Studies on the cAMP generating system in rat cerebral cortex slices using 6FNE as a specific α-adrenergic agonist have led to a clearer understanding of the role of the α-adrenergic receptor in cAMP release. Thus, it has been demonstrated that the α-receptor does not stimulate cyclase, but instead potentiates the response of cyclase to endogenous adenosine.

Additional analogs of adrenergic agonists have been synthesized in order to try to define more clearly the "fluorine effect" on agonist specificity.

Unlike NE, N-isopropylnorepinephrine (isoproterenol) is a relatively pure β-adrenergic agonist. 2- and 6-Fluoroisoproterenol have been prepared and investigated in Guinea Pig heart preparations. Initial results indicate the α-fluoro isomer is a somewhat better β-agonist than isoproterenol while the 6-fluoro derivative shows both α and β-adrenergic agonist activity, thus providing another example of an α-shift mediated by a fluorine substituent in the 6-position. To extend these studies, analogs of phenylephrine (a relatively pure α-adrenergic agonist) have been synthesized having fluorine in the 2- or 6-positions. Results of testing of these analogs will have added significance because of the presence of only one phenolic group on the aromatic ring. To determine the effects of other substituents on NE, 2, and 6-chloro NE have been prepared.

6-Fluoro-α-methylDOPA has been synthesized and has been shown to be effective (comparable to α-methylDOPA) in lowering the blood pressure of spontaneously hypertensive rats.

^{19}F NMR Spectra as Probes of Molecular Motion

^{19}F NMR studies of fluorinated molecules in intact platelets have continued. It has been determined that human and pig platelets differ in the mechanism of amine storage (magnesium versus calcium associated, respectively). In pig vesicles, both ATP and serotonin (as judged from the behavior of difluoroserotonin) appear to exist in a gel-like state, whose molecular tumbling rates increase linearly (by about two orders of magnitude) as the temperature is raised from 4°C to 37°C. In human platelets, adenine nucleotides and pyrophosphate appear equally restricted in motion at 4°C and 37°C. Loss of difluoroserotonin from the cell at temperatures above 22°C precludes determination of restricted motion of this molecule at higher temperatures. [K. L. Kirk, J. L. Costa (NIMH), C. M. Dobson (Harvard U.), F. M. Poulsen (Harvard U.), C. R. Valeri and J. J. Vechione (Naval Blood Research Lab., Boston)]

Significance to Bio-medical Research and the Program of the Institute: Biogenic amines play a key role in neurotransmission, metabolism, and in control of various physiological processes. Relatively little is known regarding mechanisms of transport, storage, release, and action of these agents. The availability of effective analogs provides a unique set of tools for the study of these mechanisms.

Proposed Course: Additional analogs will be synthesized to help answer specific questions regarding the biochemistry and pharmacology of biogenic amines. Evaluation of the analogs will be continued with a view to development of possible therapeutic agents.

Publications:

J. L. Costa, C. M. Dobson, K. L. Kirk, F. M. Poulsen, C. R. Valeri, and J. J. Vechione: Nuclear Magnetic Resonance Studies of Blood Platelets. Proc. Royal Soc., Series B (in press).

J. E. Martin, K. L. Kirk, and D. C. Klein: Effects of 6-Hydroxy-, 6-Fluoro- and 4,6-Difluoromelatonin on the In Vitro Pituitary Response to Luteinizing Hormone-Releasing Hormone. Endocrinology (in press).

K. L. Kirk, D. Cantazuzene, Y. Nimitkitpaisan, and C. R. Creveling. Synthesis and Biological Properties of 2-, 5-, and 6-Fluoronorepinephrins. J. Med. Chem. (in press).

K. L. Kirk: A Convenient Synthesis of 3-Fluoro-L-tyrosine and 3,5-Difluoro-L-tyrosine. J. Org. Chem. 10, 2015-2016, 1980.

L. I. Goldberg, J. D. Kohli, D. Cantacuzene, K. L. Kirk, and C. R. Creveling: Effects of Ring Fluoroination on the Cardiovascular Actions of Dopamine and Norepinephrine in the Dog. J. Pharmacol. Exptl. Therapeut. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19801-35 LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Service Functions and Instrumentation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

	PI: D. F. Johnson	Section Chief	LC-NIAMDD
Other:	H. J. C. Yeh	Research Chemist	LC-NIAMDD
	W. R. Landis	Chemist	LC-NIAMDD
	P. Parisius	Chemist	LC-NIAMDD
	B. Baer	Chemist	LC-NIAMDD
	A. Wong	Chemist	LC-NIAMDD
	N. Whittaker	Chemist	LC-NIAMDD
	B. Torain	Biol. Lab. Tech.	LC-NIAMDD

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Chemistry

SECTION
Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	7.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed included organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses included: GC/MS spectrometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, amino acid analysis, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

PHS-6040
(Rev. 10-76)

Project Description: Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses include: GC/MS spectrometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrometry, amino acid analysis, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses performed in the microanalytical laboratory was 17,972 an increase of 40% since the last reporting period. Review of the special types of analyses are shown below:

	Analyses Run		
	1978-79	1979-80	% Change
Carbon	2398	2480	3.4
Hydrogen	2398	2480	3.4
Nitrogen	3886	4288	10.3
Phosphorous	310	193	-37.7
Sulfur	88	119	35.2
Halogens	270	314	16.3
Metals	36	50	38.9
Weight Loss	45	52	15.6
Rotations	35	54	54.3
Miscellaneous	328	313	-4.6
Protein (Technicon)	3072	7629	148.3
	12,866	17,972	39.7

Under a continuing contract with Galbraith Laboratories 30 special samples were submitted for a total of 95 analyses. The distribution of service rendered was LC/LBC (47%); NIH, not including LC/LBC (48%); and outside agencies (4%). It can be seen that the analytical service is operating at a high level with a substantial increase over the last reporting period.

The amino acid analysis service is entering its second year of operation. Ms. Barbara Torain operates and maintains two Beckman 121 MB instruments, analyzing both hydrolysates and physiological samples. A total of 535 samples (350 protein hydrolysates - 185 physiological) were run, representing an increase over the past year. The requests by Institute were; NIAMDD (272), NIAID (18), NCI (116), NINCDS (31) and NHLB (98). This recently instituted service is fulfilling the expectation of providing occasional

analyses for large number of investigators obviating purchase of expensive equipment for occasional use.

The NMR service includes a Varian A-60, HA-100, and JEOL FX-100 pulse Fourier transform spectrometers. As in the last reporting period approximately 150 spectra were run on the A60 by Mr. Benjamin Miller and over 1000 by individual investigators. Mr. Miller continues to maintain the documentation, ordering, and stocking of organic chemical stores for Building 4. A total of 400 spectra were run on the JEOL FX-100 as a service function by Dr. Herman Yeh. These specialized spectra were in addition to Dr. Yeh's research program, reported elsewhere. This represents a 36% increase of service workload for Dr. Yeh

A total of 73 samples were submitted to Mr. William Landis for mass spectral analysis on the Hitachi Perkin-Elmer RMU-6E. Analyses involved several spectral determinations. Each sample analysis was successfully completed according to the investigator's requests and the instrument's capability. This involved standard qualitative spectra; isotope quantitative mass-ratio measurements; on-scale spectra recorded either/or the visicorder and the pen recorder; and sample mixtures requiring fractional volatility- changing rate separations occurring in the solids-inlet probe.

The number of samples analyzed are tabulated as follows:

NIAMDD - Laboratory of Chemistry	907
NIH, other than NIAMDD-LC	94
Outside NIH	74
(Total samples analyzed)	72
	1073

Mr. Noel Whittaker is the principal operator of the Finnigan GC/MS and VG 70-70 systems. The latter, a recent acquisition is in full operation; GC/MS, chemical Ionization/GC/MS in both nominal mass and accurate mass modes. Over 1000 spectra were run for LC and LBC primarily with an additional 200 spectra for investigators other than LC/LBC.

The Section Chief continues to serve as Project Officer on an NIAMDD contract with the Medical Research Council of England. This contract provides reference steroid compounds to qualified research workers in the U.S.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19802-07 LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Characterization of the Dehydrogenase-Isomerase Enzymes and Metabolic Pathways in Tetrahymena Pyriformis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nancy Lamontagne	Research Chemist	LC-NIAMDD
Other:	David F. Johnson	Section Chief	LC-NIAMDD

COOPERATING UNITS (if any)

Chester E. Holmlund, Dept. of Chem., Univ. of Md., College Park, Md.

LAB/BRANCH
Laboratory of Chemistry

SECTION
Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our objective is to isolate a dehydrogenase from a cell-free extract of T. pyriformis. The partially purified enzyme demonstrates a substrate specificity for secondary alcohols, therefore the kinetics with both non-steroidal alcohols will be studied. Characterization of the secondary alcohol dehydrogenase will include pH, optimum molecular weight, effect of inhibitors and heat. A growth-inhibition study will also be done to investigate whether the in vivo reduction of the ketone at the C-3 of a steroid is a detoxication mechanism.

PHS-6040
(Rev. 10-76)

Project Description: The protozoan Tetrahymena pyriformis has steroid dehydrogenases that are similar in functional properties to the dehydrogenases of higher eukaryotes. Specifically, our prior identification of 4-androstene-3β,17β-diol and pregnenolone as transformation products from the incubation of T. pyriformis with testosterone and progesterone, respectively, indicated that this protozoan possesses a dehydrogenase-isomerase system that can isomerize a Δ^4-steroid to a Δ^5-steroid and reduce the C-3 carbonyl to a 3β-hydroxyl. The characterization of the dehydrogenase is being pursued.

The secondary alcohol dehydrogenase (SADH) is obtained as a crude supernatant from a 100,000 x g centrifugation of ruptured cells. Purification steps are DEAE-cellulose, ammonium sulfate precipitation, affinity chromatography on Cibacron Blue F3G-A Sepharose, and chromatography on Sephadex G-100. The amount of enzyme activity is low, requiring multiple preparations of the early purification steps to provide sufficient activity to measure in the last step.

The dehydrogenase activity with steroidal and non-steroidal substrates co-purifies. Other evidence supporting the hypothesis that both activities reside in the same protein include: similar heat denaturation behavior; similar behavior with potential inhibitors; and the same pH optimum.

Kinetic studies are limited by the low solubility of the substrates, but the apparent K_m and V_{max} are being determined for a number of steroidal and aliphatic alcohols.

Characterization of the SADH will include pH optimum, molecular weight, isoelectric point and heat inactivation.

Cultures of T. pyriformis will be grown in the presence of varied concentrations of unlabeled steroids. Growth inhibition will be measured by cell counting in a hemocytometer. A fixative technique has been developed to immobilize the protozoa and Trypan blue will be used to distinguish between viable and nonviable intact cells.

Publication: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19803-07 LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Applications of NMR in Biochemical and Biological Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. C. Yeh	Research Chemist	NIAMDD-LC
Other:	D. F. Johnson	Section Chief	NIAMDD-LC
	S. Chiang	Visiting Fellow	NIAMDD-LC
	C. L. Chatterjee	Visiting Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

S. M. Wang and C. C. Yang (Nat'l. Tsing Hua Univ., Taiwan)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop nuclear magnetic resonance methods for elucidating molecular structure and for studying the interactions within and between molecules, especially those of biological interests.

PHS-6040
(Rev. 10-76)

Project Description: Basic information on the structure and molecular interactions of biologically important materials is of significance to biochemical and biological research. One of the most promising approaches in obtaining such information is through the use of nuclear magnetic resonance. The purpose of this project is to develop and apply nuclear magnetic resonance methods for studying molecular structures and molecular interactions in biochemical and biological systems.

Hemerythrin, a non-heme oxygen-carrying protein found in the erythrocytes of certain marine invertebrates, normally occur in an octameric form of molecular weight of 108,000 daltons, and can be dissociated into the monomeric species in the presence of several organic mercurials. Each subunit contains two atoms of iron and can reversibly bind one molecule of oxygen. Oxidation of either oxyhemerythrin or deoxyhemerythrin gives the iron (III) species, methemerythrin, which coordinates a number of simple anionic ligands to give a series of characteristic methemerythrin complexes.

In order to gain information on the conformation and the nature of intersubunit interaction of the protein, we have used NMR spectroscopy to examine the titration behaviors of histidine ring C-2 proton resonances of P. goudii hemerythrin and its subunit A and B, and myohemerythrin from T. zostericola with a number of ligands. Spectral comparison of the monomeric hemerythrins obtained from these species has enabled us to assign several histidine resonances and to characterize their local structures. These include two histidine resonances in hemerythrin A (His-82 and His-34) and one each in hemerythrin B (His-34) and myohemerythrin (His-57). The readily titrable His-82 (pK 6.5) in hemerythrin A and His-57 (pK 6.4) in myohemerythrin in the pH range 4 to 9.5 suggest that these histidines probably are exposed near the surface of the protein. That His-34 in hemerythrin A and hemerythrin B can be observed only below pH 4.5 indicates that His-34 may be partially buried inside the protein or hydrogen-bonded to nearby amine acid residues. The results eliminate the possibility of His-32, His-34 and His-57 as ligands to the iron atoms and hence imply that a maximum number of five histidines are coordinated to the iron atoms.

We have examined the histidine C-2 proton titration behaviors of both octameric and monomeric methemerythrins with several anions (e.g. N_3^-, SCN^-, Cl^-, CN^-, and F^-). The titration-data for His-34 were found noticeably different between ligands such as N_3^- (or SCN^-) and Cl^- (CN^- or F^-), and pKa values of His-34 were found considerably higher for the monomeric than the octameric methemerythrins. These findings tend to provide evidence of conformational differences among these hemerythrin complexes and the involvement of His-34 in the intersubunit interactions.

Neurotoxins from snake venoms are a group of small proteins which block the neuromuscular transmission at the post-synaptic membrane by high affinity binding to acetylcholine receptors and accordingly are a subject of considerable interest in the field of neurochemistry. The toxins show a high degree of sequence homology and 13 amino acid residues are found to be invariant among more than 50 neurotoxins being investigated. Considerable attention has been

devoted to the investigation of the roles played by some of the amino acid residues in the binding to the receptor and in the lethal activity of the toxins. Basic information on the conformation and structure of the toxins in solution is of importance to the understanding of the nature of toxin-receptor interaction. Although the main chain conformation of these proteins has been elucidated in some detail, relatively few investigations on the local structures of the amino acid residues of the toxins have been reported. In order to gain understanding of the structure-toxicity relationship for these toxins we have undertaken an exploration of the local structure of the toxins using nuclear magnetic resonance as a tool in assessing the detailed structural information of these toxins.

The results of our preliminary ^{1}H and ^{13}C NMR work on cobrotoxin and pelamitoxin have revealed some detailed structural information, including the micro-environments of His-4, Tyr-25 and His-32 of cobrotoxin and His-30 and His-54 of pelamitoxin. The microenvironments of the two histidines in both cobrotoxin and pelamitoxin are found different as reflected from the differences in the chemical shifts of the C-2 ring protons and pK_a values of these histidines. In combination with deuterium and tritium exchange experiments, which utlilize the difference in isotope exchange rates of the imidazole C-2 hydrogens between the two histidines, the two titratable resonances at δ=7.7 and 7.8 ppm at pH=9.0 for cobrotoxin are assigned to the C-2 ring protons of His-4 (pK_a=5.1) and His-32 (pK_a=5.7), respectively. The readily titratable property of both histidines indicates that they are accessible to solvent water molecules. That the pK_a value of His-4 is lower by 0.6 unit than that of His-32 suggests the presence of a positively charged amino group(s) near His-4. A significant difference in the conformation between the two histidines is also evidenced from the pH-dependence of the ^{13}C chemical shifts of their imidazole carbons. His-4 is found to exist predominently as a 1,5 tautomer, while His-32 as 1,4 tautomer. A detailed structural study of these toxins, including some chemical modification, is now in progress.

Publication: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19804-07 LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Development of Chemical Methods and Compounds for the Study of Biological and Medical Problems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Calvin M. Foltz	Research Chemist	NIAMDD-LC
Other:	Lance A. Liotta	Expert/Pathologist	NCI-LPP

COOPERATING UNITS (if any)

National Cancer Institute

LAB/BRANCH

Laboratory of Chemistry

SECTION

Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The primary goal of this work is to contribute to the investigation and solution of basic biological and medical problems. This is being done by the application of chemistry to the isolation and study of biomolecules, by the synthesis and study of chemical compounds, and by the study of the interactions of chemicals and biologicals with cells, such as those of the TU24l sarcoma and B16 melanoma, in vitro and with tumor cells and tumors in mice. Areas of special interest are organic chemistry, photochemistry, selenium chemistry, medicinal chemistry, biochemistry, cell biology, tissue culture, and cancer chemotherapy.

PHS-6040
(Rev. 10-76)

Project Description: The objective of this project is to make contributions to the investigation and solution of basic biological and medical problems by the application of chemical, physical, and biological methods.

The focus of present work is the study of cancer metastases in the mouse. The purposes of this study are: (a) to increase our understanding of the mechanisms operating at the various stages in the formation of metastases, (b) to study the effects of chemicals and biologicals on metastases and their development, and (c) to apply the knowledge obtained thereby to the design and synthesis of organic compound of potential use in inhibiting the formation of metastases or selectively destroying them in human beings. The findings of these studies may also contribute to the study of other biological and medical problems.

The population of cells in most malignant tumors is thought to be heterogeneous. The subpopulation of cells which is most invasive and metastatic may be of special value in the study of the formation of metastases. After fifteen cycles of transplanting cells of lung metastases of the PMT subline of the TU241 sarcoma to muscle in C57Bl/6N mice no increase in metastatic potency of the tumor was found and the study was terminated. The result indicates that the metastatic potency of this tumor cannot be increased by this method and that it may already be maximal. The fact that these cells cannot be grown in vitro is probably also a manifestation of this high metastatic potency.

A neutral protease has been isolated from cultures of the highly invasive and metastatic TU241-PMT cells freshly isolated from the tumor. This enzyme preferentially degrades type-IV collagen, the principal collagen of basement membrane, and may play a decisive role in the invasiveness and metastatagenicity of the tumor. Studies of metastasis formation in the mouse and measurements of the type-IV collagenolytic activity show a correlation of the metastatic potency of B16-F1, B16-F10, B16-BL6, and TV241-PMT cells with this type of enzyme activity. Human leukocytes and MCF-7 human breast carcinoma cells also possess the activity, but mouse fibroblasts and bovine endothelial and aorta smooth muscle cells do not. If this enzyme activity does play a critical role in tumor invasiveness and metastasis formation, it may afford a route to interference with the process of metastasis formation.

A method has been developed for the measurement of basement membrane collagen degradation by tumor cells in vitro. (^{14}C)Proline-labeled type-IV collagen synthesized by EHS sarcoma cells is used to coat 16mm wells of tissue culture clusters. Cells in appropriate media are incubated in the wells and the radioactivity of the fragments of labeled substrate released into the medium is counted. Correlation of metastatic potency with type-IV collagenolytic activity is also indicated using this method. Data obtained by this method also suggest that the mechanism of degradation is local activation of a type-IV collagen-specific enzyme secreted at the cell-substrate interface with no significant phagocytosis of the substrate.

A rabbit antiserum to the type-IV collagenolytic activity has been produced. The antiserum shows slight cytotoxicity to TU241-PMT cells in vitro. Such

cells, isolated from the tumor and in single cell suspension, after incubation with the antiserum and intravenous injection produced very few lung metastases in the mouse; whereas, the incubated but untreated control cells were very highly metastatic. Work on the preparation of the antibody and its study is in progress.

Publications:

Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, Ian, Foltz, C. M., and Shafie, S.: Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature, 284, 67-68 (1980).

Foltz, C. N., Garbisa, S., and Liotta, L. A.: A method for the quantitation of basement membrane collagen degradation by metastatic tumor cells in vitro. Am. Assn. Cancer Res. Proc. 71st Ann. Mtg., 21, 27 (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19806-05 LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Nature of Steroid Receptor Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. S. Simons, Jr.	Senior Staff Fellow	NIAMDD-LC
Other:	D. F. Johnson	Section Chief	NIAMDD-LC
	M. Pons	Visiting Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

E. I. B. Thompson, NCI, L. D. Loriaux, NICHD, Herman Ammon, Univ. of Md. Chem. Dept.

LAB/BRANCH
Laboratory of Chemistry

SECTION
Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

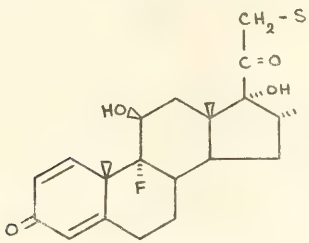
SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to define the initial, intra-cellular events of steroid hormone action. Synthetic glucocorticoid derivatives, some of which could react to form covalently labelled receptors via affinity labelling, are being used with glucocorticoid-responsive rat hepatoma tissue culture cells to examine: (1) steroid-receptor binding site interactions; (2) the effects of steroid binding on receptor conformation; and (3) the nature of "activation" of receptor-steroid complexes. Fluorescence spectroscopy represents one technique for examining these interactions. Two series of synthetic steroids have been synthesized which potentially could give covalent, fluorescent receptor-steroid complexes via fluoroscent chemo-affinity labeling. In another approach, we have incorporated the novel reactive functional group β-hydroxy-α-keto-mesylate into cortisol to yield cortisol-mesylate, which appears to be the first long acting, and possibly irreversible, anti-glucocorticoid.

PHS-6040
(Rev. 10-76)

Project Description: Steroid specific events are induced by nanomolar concentrations of hormone in responsive cells. Thus, extremely sensitive methods are required in order to monitor the steroid at physiological levels. Fluorescence spectroscopy can 1) detect some compounds at concentrations as low as 10^{-12} M and 2) provide information about the microenvironment of the fluorescent molecule. These two properties make fluorescence spectroscopy ideally suited for a study of steroid-receptor interactions. Due to the tendancy of receptor-steroid complexes to dissociate, a covalently bound fluorescent receptor-steroid complex would be even more useful. In order to obtain such a complex, we have recently developed a new methodology for affinity labelling which we call fluorescent chemo-affinity labelling (FCAL). In FCAL o-phthaladehyde specifically cross-links primary amines and thiols to give strongly fluorescent 1-alkylthio-2-alkylisoindoles in high yields. In collaboration with Herman Ammon of Univ. of Md., we are trying to obtain an x-ray structure of an isoindole derivative in order to unambigously prove the isoindole structure and to answer some basic theoretical questions about this ring system.

In order to obtain a fluorescent, covalent receptor-steroid complex, we have prepared two series of hydrolytically stable glucocorticoid derivatives (1 and 2) which should participate in the FCAL reaction. In model studies with OPTA



1.) X = SH (n = 2-6)

2) X = NH_2 (n = 2-6)

and an added amine or thiol, each steroid gives a good yield of the desired fluorescent isoindole. In collaboration with Dr. Thompson, we found that each steroid displays high affinity for the glucocorticoid receptors from rat liver hepatoma tissue culture (HTC) cells and induces the enzyme tyrosine aminotransferase (TAT). Thus these synthetic steroids show excellent promise for being effective FCA labels for glucocorticoid receptors. Experiments aimed at detecting these postulated, covalent, fluorescent receptor-steroid complexes are in progress.

Following another, more conventional approach, we have tried to develop new reactive functional groups which could be used as electrophilic affinity labeling groups (see also previous reports). A systematic study of the reactivity of α-keto mesylates with various nucleophiles (i.e., carboxylate, -OH, imidazole, $-NH_2$, thiol acid anion and -SH) under mildly basic conditions revealed that α-keto mesylates do not react with imidazole or hydroxyl groups, react extremely slowly (if at all) with carboxylate and primary amines, and react several thousand times faster with thiols and thiol acid anions. The

very rapid reaction with thiols occurs only with the dissociated thiolate anion. The addition of a β-hydroxyl group to α-keto mesylates accelerates the reaction with thiolate anions by a factor of 3-12 in acetone but has no effect on reactions run in dimethylformamide. α-Keto mesylates exhibit the same selectivity for thiolate anions, as compared to amines, as do α-keto chlorides. In view of this reactivity and selectivity, the α-keto mesylate appears to be a promising functional group for the electrophilic affinity labeling of biological macromolecules in weakly basic solutions.

Cortisol-21-mesylate (CM) was prepared in hopes of obtaining an alkylating derivative of cortisol. The biological properties of CM were investigated in HTC cells. CM appears to bind to glucocorticoid receptors in cell-free extracts since CM inhibits the specific binding of ^{3}H-dexamethasone. However, in whole cells, CM not only fails to induce the enzyme TAT but also inhibits the induction of TAT by dexamethasone. Thus CM is an anti-glucocorticoid. This is not due to cell death since CM is relatively non-toxic up to concentrations of 10^{-5}M. The concentration of CM needed for half maximal inhibition of TAT induction is an order of magnitude lower than that predicted from the apparent, cell-free affinity of CM for the glucocorticoid receptors of HTC cells, thus suggesting that the cell-free binding data does not reflect an equilibrium situation. In fact the reactive α-keto mesylate group was intentionally incorporated into cortisol in hopes of obtaining a steroid capable of undergoing chemically irreversible reactions. When HTC cells are pre-incubated with either CM or the reversible anti-glucocorticoid progesterone and then washed to remove free steroid, only the CM treated cells fail to show subsequent induction of TAT by dexamethasone. Furthermore, pre-incubation of HTC cell cytosol with CM blocked ~75% of the subsequent exchange binding of ^{3}H-dexamethasone to glucocorticoid receptor sites. Thus, the actions of CM in whole and broken cells either require an exceptionally long time for reversal or are not reversible. Together, these results indicate that CM is a unique antagonist and could be the first irreversible anti- glucocorticoid *in vitro*.

We are applying organic chemistry to the study of an important biological molecule(s) - the intracellular steroid hormone receptor(s). A molecular understanding of steroid-receptor interactions is highly desirable, especially since the therapeutically valuable, steroid-specific differences are most likely expressed at this level of steroid hormone action.

Publications: Simons, S. S., Jr.: Factors influencing association of glucocorticoid receptor-steroid complexes with nuclei, chromatin and DNA: Interpretation of binding data. In Baxter, J. D. and Rousseau, G. G. (Eds): *Glucocorticoid Hormone Action*. Springer-Verlag, Berlin, p. 161-187 (1979).

Simons, S. S. Jr., Thompson E. B., and Johnson, D. F.: Fluorescent chemo-affinity labelling: Potential application of a new affinity labelling technique to glucocorticoid receptors. *Biochem.*, *18*, 4915-5922 (1979).

Bloom, E., Matulich, D. F., Lan, N. C., Higgins, S. J., Simons, S. S., Jr., and Baxter, J. D.: Nuclear binding of glucocorticoid receptors: relations between cytosol binding, activation and the biological response. J. Steroid Biochem., 12, 175-184 (1980).

Simons, S. S., Jr., Thompson, E. B., Merchlinsky, M. J. and Johnson, D. F.: Synthesis and biological activity of some novel, chemically reactive glucocorticoids. J. Steroid Biochem., 13, 311-322 (1980).

Thompson, E. B., Simons, S. S. Jr., and Harmon, J. M.: Deacylcortivazol, a potent glucocorticoid with unusual structure and unusual antileukemic cell activity. Hormones and Cancer Symposium (Worcester Foundation) Plenum Press, in press.

Simons, S. S., Jr., Pons, M. and Johnson, D. F.: α-Keto mesylate: A reactive thiol-specific functional group. J. Org. Chem., in press.

Simons, S. S., Jr., Thompson, E. B., and Johnson, D. F.: Unique long acting anti-glucocorticoid in whole and broken cell systems. Proc. Natl. Acad. Sci. USA., in press.

ANNUAL REPORT
LABORATORY OF EXPERIMENTAL PATHOLOGY

The Laboratory of Experimental Pathology has continued a variety of research projects.

The Office of the Chief conducts work for the whole NIAMDD. The services which are major to assets to the institute include electron microscopy (EM) and histopathology. During the last year Dr. Bladen's unit has been involved with EM services for several NIAMDD and other NIH investigators involving both long term studies and screening of samples. The histopathology tissue laboratory under Ms. Reveal has continued to provide technical assistance to the investigators of the Laboratory of Experimental Pathology and to those of other units at the NIH who require such technical help. A considerable part of the tissue laboratory's work is concerned with providing a wide variety of special histologic techniques adjusted to the various research projects as shown below:

	Specimen Accessioned	Stained slides Routine	Stained slides Special	Spare Slides	Total
Animals	176	232	1240	2102	
Human	4	6		6	
	180	238	1240	2108	
		1478			
					3586

The Section on Chemical Pathology has continued work in virology, cell biology, and tissue culture. Much of this work has concerned interferon and the molecular biology of DNA virus replication and their implications for cell biology.

The work on interferon has included studies on its mechanism of action and its effects on cell-free protein synthesizing systems and on cells in culture. A 200-fold inhibition in the titer of infectious vesicular stomatitis virus (VSV) was achieved in cultures of L-cells treated with 30 reference units/ml of interferon; however virus particle production, as measured by VSV particle-associated RNA, nucleocapsid (N) protein, or transcriptase activity, was inhibited by a maximum of 10-fold by this concentration of interferon. A significant reduction was seen in the glycoprotein (G) and matrix protein (M) content of VSV from interferon-treated cells. Electronmicroscopic studies also support the deficiency of glycoprotein in VSV released from interferon or tunicamycin-treated cells. The incorporation of N-acetylglucosamine into glycolipids with the properties of dolichol derivatives is lower in membranes prepared from interferon-treated cells as compared to the untreated cells. These results suggest that interferon inhibits the glycosylation of a viral protein in this system.

We have also previously shown that interferon alters the structure of the plasma membrane. Furthermore, both Moloney murine leukemia virus and vesicular stomatitis virus (VSV) produced by IF-treated cells have low infectivity. While the content of the G protein (envelope glycoprotein) in VSV produced in IF-treated cells is reduced, no defect in the net charge or molecular weight or glycosylation and silialyation of the G protein was observed. We have also studied the biosynthesis of the viral proteins (VP's). Although IF inhibits the synthesis of VPs to a varying extent depending on the dose, this inhibition is not likely to be responsible for the reduced incorporation of G protein into the virions since the intracellular VPs accumulated is in vast excess of that incorporated into the virion. The relative rate and extent of glycosylation of intracellular G protein in the IF-treated cells are not changed. The decreased amount of G protein in the low infectious virion may be due to blocks in the intracellular transport or utilization of G protein in the packaging of the virion on the plasma membrane.

The aim of the interferon research in cell-free protein synthesizing systems is to determine the biochemical basis of interferon action. Work has focused on 2'5'oligoadenylic acid synthetase and a protein kinase activity which phosphorylates a 67,000 dalton protein, two enzymatic activities which are induced by interferon and enhanced by some double-stranded ribonucleic acids (dsRNA). The work has been divided into two studies: 1) The relationship of polynucleotide structure to the activation of 67K protein kinase activity and protein synthesis inhibition; and, 2) the role of oligo A synthetase in the establishment and maintenance of the anti-viral effects of interferon.

Other studies have involved the purification of interferon messenger RNA in studies aimed ultimately at cloning a human gene for interferon production. The human messenger RNA is obtained from lymphoblastoid from NAMALWA cells. Partial purification of this mRNA has led to about a 90-fold enrichment in our best preparations. The enriched preparations are transcribed by an RNA tumor virus reverse transcriptase and double stranded DNA is made from this.

Other studies in the Section on Chemical Pathology have employed DNA viruses as molecular probes to elucidate genome expression in human cells. We are studying intensively the structure and function of adeno-associated virus (AAV) since this virus has only a small genome. We are studying also adenovirus which is a more complex genome. Adenovirus is the helper virus for AAV multiplication. This helper relationship is being analysed. Also both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus this causes malignant transformation of the cell. AAV inhibits this transformation and also inhibits Ad12 oncogenesis in newborn animals. Thus AAV inhibits tumor induction. The mechanism of this inhibition of tumor induction is being studied at the molecular level in both cell culture and in animal experiments.

The section on cytogenetics has carried out both clinical and research studies including studies of patients and their families with certain genetic

disorders due to chromosomal abnormalities or single gene defects. Techniques used include newly-developed chromosomal banding and differential staining, analysis of genetic markers such as enzymes or HLA within pedigrees, and dermatoglyphic analysis. Findings are correlated with phenotypic abnormalities and may be utilized for genetic counseling. They have also carried out karyotypic evaluation of tissues from laboratory animals which are models of different human genetic disorders, primarily primate and murine. Relationship of cytogenetic variation to defects associated with certain diseases is stressed. Effects of gross chromosomal abnormality upon reproductive fitness and growth and development are also studied.

Other projects in this section have included: application of differential staining techniques for chromosome analysis; studies on the inheritance of several autoimmune immunological traits including antibodies cytotoxic for T lymphocytes and reactive with single stranded DNA; studies on hyperdiploidy inheritance; analysis of sex hormone modulation of immune responses; analysis of aneuploid cells by flow cytometry; and studies of sex hormone receptors in the murine thymus.

The Section on Molecular Pathology has continued studies on the chemistry and structure of amyloid and amyloid related proteins. The main topics of present interest are: isolation and chemical characterization of amyloid fibril protein from various types of systemic amyloidosis and localized amyloid deposits; mechanism of formation of amyloid deposits from serum precursors; isolation and purification of proteolytic enzymes involved in amyloid fibril formation; and, immunochemical characterization of amyloid fibrils from different syndromes in tissue section for diagnostic and therapeutic purposes.

The Section on Biophysical Histology has concentrated this year on neurobiology and morphology. Studies on the genetics of nerve cell shape have employed the snail B. glabrata and the dye Lucifer Yellow in conjunction with electron microscopy.

The Section on Cellular Function and Ultrastructure has carried out a varied program on cell biology and biophysics. They have studied the distibution of enzymes in the pregnant rat uterus and developing placenta using immunocytochemical methods. In addition, an improved version of earlids, the active hearing protectors has been built and other studies indicate that when any Hookean material is deformed, the losses equal the ultimate strain energy. Using this relation, it has been shown that the time lag in the response of poroelastic material such as cartilage is roughly proportional to the square of the lenght of the path followed by the pore liquid during creep. For samples of different size but the same shape this proportionality is exact.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,001-26 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Preparation of stained tissue sections for investigation and diagnostic purposes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Virginia Reveal Head, Tissue Preparation Lab. LEP NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NIAMDD, LEP, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The tissue laboratory will continue to provide technical assistance to the investigators of the Laboratory of Experimental Pathology and to those of other units at the NIH who require technical help. A considerable part of the tissue laboratory's work is concerned with providing a wide variety of special histologic techniques adjusted to the various research projects as shown below.

	Specimen Accessioned	Stained slides Routine	Stained slides Special	Spare Slides	Total
Animals	176	232	1240	2102	
Human	4	6		6	
	180	238	1240	2108	
		1478			
					3586

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,002-15 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Cellular Function and Ultrastructure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lois W. Tice	Chief, Section on Cellular Function and Ultrastructure	LEP	NIAMDD
OTHER:	Raymond Carter	Biomedical Technician	LEP	NIAMDD
	Marjorie Cahill	Bio. Lab. Tech	LEP	NIAMDD

COOPERATING UNITS (if any)
Dr. J. W. Daly, LC:NIAMDD
Dr. C. R. Creveling; LC:NIAMDD

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Cellular Function and Ultrastructure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Changes in the distribution of COMT in the pregnant rat uterus and developing placenta were examined using immunocytochemical methods.

Project Description: Z01 AM 21,002-15 LEP

Catechol-O-methyl transferase (COMT) catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to one of the phenolic hydroxyl groups of a variety of catechols. This O-methylation reaction is important in the enzymatic inactivation of circulating catecholamines, the detoxification of xenobiotic catechols, and in the local inactivation of catecholamines released at neurotransmitters from the terminals of both central and peripheral catecholamine-containing neurons. It also is receiving increasing attention for its role in metabolism of catecholestrogens. The enzyme is present in tissues of the reproductive system including the vas deferens, oviduct, mammary gland, and uterus. The level of COMT in rat uterus, higher during estrus than diestrus, increases more than 2-fold during pregnancy.

We have examined the immunocytochemical distribution of COMT in rat uteri and in the developing placenta during the course of gestation. COMT localization was determined using a specific antibody produced in rabbits with a soluble form of COMT isolated from rat liver and the peroxidase-anti-peroxidase method described by Sternberger. Our results suggest that the enzyme may play a crucial role in regulation of estrogen levels in the pregnant rat uterus.

No specific staining was observed in uteri from nonpregnant rats during estrus or diestrus. The uterus remained unreactive for COMT until day 5 of pregnancy, when the endometrial and glandular epithelium because distinctly COMT-positive. On day 7 differentiating decidual cells in segments of the uterus near the implanting blastocyst showed COMT-positive reaction was still observed in the epithelium of glands which remained in the stroma near the myometrium. As pregnancy progressed, on day 8, the areas of positive reaction formed a crescent around the antimesometrial border of the lumen at the point where the blastocyst was in contact with the endometrium. Some endometrial glands in the stroma and at the border of the stroma were still positive.

The thin superficial region between the implantation site and the lumen, the decidua capsularis, on the antimesometrial side of the uterus, showed a positive reaction. COMT-positive cells were also present in the differentiating vascular or deciduotrophoblastic zone in the decidua basalis. In the vascular zone, two kinds of COMT-positive cells were present. One, polygonal in shape, was present in the walls of the vascular space. The other cell type was stellate in shape and bordered the vascular zone near the decidua basalis. These cells had the shape and general distribution of the so-called "metrial gland" cells.

COMT was originally thought to function primarily in catecholamine inactivation, both of circulating hormones and at postsynaptic adrenergic nerve terminals. There are at least two structurally distinct adrenergic neuronal systems present in the uterus associated with blood

vessels and with the myometrium. The catecholamine content of the uterus varies during the estrus cycle. In addition, norepinephrine and epinephrine injected into the mother are known to cross the placental barrier. There are relatively high levels of endogenous epinephrine in the uterus during pregnancy. Further, it is known that 2-hydroxyestradiol induces uterine hyperaemia, a response inhibited by alpha-adenergic blocking agents, an observation which suggests an interaction between catecholamine receptors and estrogens.

However, a number of recent studies have suggested a role for COMT in inactivation of catecholestrogens. These substances, formed by the action of estrogen 2-hydroxylase, appear to function as antiestrogens, competing with estrogen for receptors in hypothalamus and pituitary. Their methylated products possess little or no activity in this regard. The two enzymes estrogen 2-hydroxylase (present in placenta, as well as in other organs, and COMT, could serve to regulate estrogen activity in sites where they both are present. Such a function is consonant with our finding that COMT is present in decidual cells of the pregnant uterus, as well as our earlier findings that COMT was present in the epithelia of both oviduct and vas deferens. Thus, the appearance and distribution of COMT during pregnancy could be related to changes in either or both catecholamines and catecholestrogens.

However, the transitory appearance of COMT in the endometrial epithelium of the preimplantation uterus would seem quite mysterious if it metabolized only its catecholamine substrates. When its role in the metabolism of catecholestrogens is considered together with the possibility that it and estrogen 2-hydroxylase may act to regulate estrogen activity at some sites, intriguing possibilities begin to emerge. Degenerative change in endometrial epithelial cells in the rat appears to precede invasion by the trophoblast, and it is well known that endometrial epithelial cells are estrogen-sensitive. If COMT in the endometrial epithelium acts as a sink for catecholestrogens, as would be the case if estrogen 2-hydroxylase were also present, then local decreases in catecholestrogen levels produced by appearance and activity of COMT could trigger the degenerative change required for trophoblast invasion, whether or not 2-hydroxyestrogens were active forms of the hormone in those cells.

Publications:

Neuwirth, M., Daly, J. W., Myers, C. W. and Tice, L. W.: Morphology of the granular secretory glands in skin of poison-dart frogs (Dendrobatidae). Tissue and Cell. 11: 755-771, 1979.

Tice, L. W., Carter, R. and Cahill, M.: Changes in tight junctions of rat intestinal crypt cells associated with changes in their mitotic activity. Tissue and Cell. 11: 293-316, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,003-12 LEP

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biomechanics and Related Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. W. McCutchen	Research Physicist	LEP	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Section on Cellular Function and Ultrastructure

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An improved version of earlids, the active hearing protectors has been built.

It has been shown that when any Hookean material is deformed, the losses equal the ultimate strain energy. Using this relation, I have demonstrated that the time lag in the response of poroelastic material such as cartilage is roughly proportional to the square of the length of the path followed by the pore liquid during creep. For samples of different size but the same shape this proportionality is exact.

PHS-6040

Project description:

Earlids:

A new prototype active hearing protector has been built with quicker-responding automatic gain control. However, because the NIH patent department has expressed its disinterest in the idea, the earlid project has been dropped.

Automatic Stability of Aircraft:

The "No-Highway"-like warning of the need for control surfaces at the front in aircraft with automatic stability has been published.

Roughness of Cartilage Surface:

The work evaluating the roughness of cartilage surface with a cheap microscope has been reported at an international meeting and is in press.

Poroelasticity of Cartilage:

It is frequently and erroneously stated that cartilage is viscoelastic, which implies that its slow response to changes in loading is an intrinsic property of the material. At least one retardation spectrum of this "material" has been published. In fact, cartilage is poroelastic. The sluggishness of its response results mostly from the flow of fluid through its pores, and the magnitude of the time lag depends on the size and shape of the specimen. As well as in its stiffness and permeability I have demonstrated mathematically that when any Hookean material is deformed the energy absorbed by losses equals the strain energy of the ultimate deformation. Using this fact, I have shown that the time lag in response of a sample of poroelastic material is approximately proportional to the square of the lenght of the path followed by the liquid as the sample creeps. For samples that are geometrically similar to each other the proportionality is exact.

Propaganda:

A chapter on joint lubrication has been prepared for Clinics in Rheumatic Diseases: "History of the Joint" Peter Hasselbacher, M.D. Ed.

Publications:

McCutchen, C. W.: Avoiding divergent stall in control configured aircraft by using a canard arrangement. J. Aircraft. 17: 283-284, 1980.

McCutchen, C. W.: Joint lubrication in the Second Munich Symposium on biology of connective tissue: Biology of articular cartilage. Ed. H. Gastpar (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,004-06 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Possible Mechanisms of Persistence of Vesicular Stomatitis Virus in Mouse L Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert M. Friedman	Chief, Laboratory of Experimental Pathology	LEP	NIAMDD
OTHER:	Janet M. Ramseur	Research Biologist	LBV	NIAID

COOPERATING UNITS (if any)
NIAID

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SECTION
Section on Chemical Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 21,005-13 LEP

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Histochemistry: Principles, Methods & Applications

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ned Feder	Chief, Section on Biophysical Histology	LEP	NIAMDD
OTHER:	Walter Stewart	Research Physicist	LEP	NIAMDD
	Kent Keyser	Staff Fellow	LEP	NIAMDD

COOPERATING UNITS (if any)

Dr. Raymond F. Chen (NHLBI)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Section on Biophysical Histology

INSTITUTE AND LOCATION

NIAMDD:NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	3	3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continuing studies with the dye, Lucifer Yellow, have been carried out in conjuction with electron microscopy.

Studies have continued on the genetics of nerve cell shape using the snail Biomphalaria glabrata as an experimental animal.

PHS-6040

Project description:

Studies of Neuron Structure and Physiology With Lucifer Yellow:

Further work has been done with Lucifer Yellow, an intensely fluorescent dye devised in this laboratory for intracellular marking of individual nerve cells.

A method for observing the same dye-injected cell by both light and electron microscopy has shown some further interesting features of neuron structure. For example, the Leydig cell of Hirudo medicinalis has been examined; the neuronal soma and some processes were found to contain many distinctive dense-cored granules similar in size and appearance to granules of recognized neurosecretory cells.

Genetics of Nerve Cell Shape:

Studies have continued on the genetics of nerve cell shape, using as an experimental animal the snail Biomphalaria glabrata. B. glabrata was chosen because of its suitability for neurophysiology, neuroanatomy, genetics, and convenient rearing in the laboratory. Our preliminary findings are as follows. (a) Over 170 presumably isogenic lines of B. glabrata have been produced by inbreeding. (b) Gross anatomical and pigmentation variants have been identified, and seven were found probably to be controlled by alleles at a single locus. (c) Identified neurons are being studied by several methods: injection with Lucifer Yellow, electrophysiology, histochemistry (including neurosecretion), and electron microscropy. (d) Several anatomical variants of the nervous system have been recognized, and studies of the anatomy, neurophysiology, and genetics of these variants are in progress. No significant findings have been made.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21006-12 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Protein and Proteolytic Enzyme Interrelationships in Normal and Disease States.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George G. Glenner	Chief, Section on Molecular Pathology	LEP	NIAMDD
OTHER:	Shigeyoshi Fujihara	Visiting Fellow	LEP	NIAMDD

COOPERATING UNITS (if any)
Dr. J. Henry, AFIP: Neuro
Dr. W. K. Engel, N:MN
Dr. F. K. Mostofi, AFIP:GU
Dr. K. Earle, AFIP: Neuropath.
Dr. M. Furusata, AFIP:GU
Dr. D. Fair, Scripps Clinic, CA
Dr. K. Weisgraber, UCSF, CA

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3	2	1

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☐ (a) HUMAN SUBJECTS ☒(b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study the chemistry and structure of amyloid and amyloid related proteins. The main topics of present interest are:

1) Isolation and chemical characterization of amyloid fibril protein from various types of systemic amyloidosis and localized amyloid deposits.

2) Mechanism of formation of amyloid deposits from serum precursors.

3) Isolation and purification of proteolytic enzymes involved in amyloid fibril formation.

4) Immunochemical characterization of amyloid fibrils from different syndromes in tissue section for diagnostic and therapeutic purposes.

A Method for Identifying Different Amyloid Fibril Types in Tissue Sections:

The differentiation of types of amyloid proteins has recently developed increased importance because of the suggestion that selective therapeutic approaches are feasible. Treatment with dimethylsulfoxide (DMSO) and/or colchicine have been reported to favorably affect AA type amyloid, while immunoglobulin type amyloid may be better treated with cytotoxic agents: e.g. melphalan. We have employed the unlabeled antibody peroxidase-antiperoxidase (PAP) method of Sternberger for identification of different biochemical types of amyloid fibril proteins on conventionally fixed, paraffin-embedded, tissue sections. The PAP method is estimated to be 100 to 1,000 times more sensitive than immunofluorescence. Although many, if not most, of the antigenic determinant sites in a given antigen are probably destroyed by routine tissue processing, the PAP method posesses sufficient sensitivity to detect small numbers of antigenic groups that resist fixation and embedding. Thus, routinely-fixed paraffin-embedded tissues from surgical procedures or autopsies performed years previously are available for study. In this investigation, the oldest tissue sections were cut from paraffin blocks that were prepared conventionally soon after autopsy performed 13 years before. These tissues were stained with very high signal-noise ratio. Another advantage of this method is that specimens can be examined in an ordinary light microscope and may be retained for years without significant fading of reaction product. Use of formalin-fixed paraffin sections instead of frozen tissue also permits excellent preservation and visulization of tissue structures with appropriate counter-staining.

Antisera raised against 6 M guanidine-denatured human amyloid fibrils from 4 patients of 3 different chemical fibril types (AA, Aλ, 2 Aκ's) were shown to react specifically with amyloid tissues from which the amyloid fibrils used as immunogen were obtained. In addition, anti-Aκ antisera showed idiotypic specificity; i.e. Aκ tissues were stained with anti-Aκ antiserum raised against autologous Aκ fibrils, but were not stained with anti-Aκ antiserum raised against homologous Aκ fibrils. It was, thus, demonstrated that this method could accurately, specifically and sensitively detect different types of amyloid fibril proteins in tissue sections and establishes its utility for diagnosis and therapy.

Identification of the Nature of Primary Localized Amyloidosis of the Genitourinary Tract:

Using the PAP method described above, eleven cases of primary localized amylodosis of the genitourinary tract were studied with specific antisera against different chemical types of amyloid fibril proteins (AA, Aλ, Aκ). Nine of these were shown to have Aλ antigenic fibril determinants suggesting that the fibrils of most, if not all, of these cases are of immunocytic origin. The cell processing the light polypeptide chain of immunoglobulin protein to fibrils in immunocytic amyloidosis of both systemic and localized type is believed to be of phagocytic nature, but exceptions may exist, e.g. in extramedullary plasmacytomas. In the cases of bladder amyloidosis with Aλ comprising the deposits, a pathogenetic mechanism can be postulated. Chronic and recurrent mucosal and submucosal inflammation leading to chronic cystitis causes an influx of lymphoplasmacellular elements one of which becomes monoclonal. The monoclone proliferates and secretes an aberrant light chain type which is "amyloidogenic". By means of lysosomal proteolysis by phagocytic cells or other physico-chemical means these light chains are formed into amyloid fibrils and deposited as aggregates in the bladder tissue.

Congophilic Angiopathy and Alzheimer's Disease:

The plethora of cellular and extracellular abnormalities in the cerebrum of patients with Alzheimer's presenile and senile dementia (AD) have made difficult an evaluation of the pathogenesis of the process and have obscured casual etiologic relationships. Granulovacuolar degeneration, neurofibrillary tangles composed of intraneuronal paired helical filaments, "primitive" and "mature" senile (neuritic) plaques, perivascular amyloid plaques and amyloid deposition involving vessels from arterial and venular to capillary dimensions (Congophilic angiopathy) are some of the more apparent derangements in AD. The significance of the presence of Congophilic angiopathy in AD has been inapparent due, at least in part, to a lack of satisfactory statistical data on its incidence primarily as the result of the limited series reviewed. We have documented the incidence of cerebral vascular amyloidosis (CA) in 43 cases of clinically and pathogically valid AD. In addition the relative incidence of several other pathologic characteristics of AD and their relationships with one another as well as with CA were studied. We have shown that, as has been suggested elsewhere, Alzheimer's presenile and senile dementia are related manifestations of a morphologically, but not necessarily etiologically, similar pathologic process. It appears, thus, to be a disease complex. We have also found that Concophilic angiopathy is a significant lesion occurring in 88% of all cases of AD and probably is of major pathogenetic importance in this disease. CA was not observed in the absence of AD. In addition differences in the ratio of plaques to neurofibrillary tangles were found indicating the possibility that they undergo interrelated, but inverse phases during the natural history of the disease.

All of the work done in this report has been performed during the principal investigator's assignment to the Armed Forces Institute of Pathology, the Laboratories of Dr. F. K.Mostofi (genitourinary pathology) and Dr. Kenneth Earle (neuropathology).

Publications:

G. G. Glenner: Amyloid Deposits and Amyloidosis: The β-fibrilloses. New Eng. J. Med. 302: 1283-1292, 1333-1343, 1980.

G. G. Glenner: A retrospective and overview of the investigations on amyloid and amyloidosis, the β-fibrilloses. In Amyloid and Amyloidosis, G. G. Glenner, P. P. Costa, F. Freitas (Eds.), Excerpta Medica, Amsterdam, p. 1-15, 1980.

G. G. Glenner, P. P. Costa, F. Freitas (Eds.), Amyloid and Amyloidosis, Excerpta Medica, Amsterdam, pp. 629, 1980.

G. G. Glenner: Congophilic microangiopathy in the pathogenesis of Alzheimer's syndrome (presenile dementia). Med. Hypotheses 5: 1231-1236, 1979.

S. Fujihara, J. E. Balow, J. C. Costa, G. G. Glenner: Identification and classification of amyloid in formalin-fixed, paraffin-embedded tissue sections by the unlabeled immunoperoxidase method. Lab. Invest., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,007-08 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Acute Phase Reactants and Amyloidosis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Jean D. Sipe	Research Chemist	LEP	NIAMDD

COOPERATING UNITS (if any)
Stefanie N. Vogel and David L. Rosenstreich, Lab. of Microbiology and Immunology, NIDR; Keith P.W.J. McAdam, Dept. of Med., Tufts Univ., Boston, Mass.

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NIAMDD, NIH, Bethesda, Maryland 20205

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0	0	0

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,008-14 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Cytogenetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. H. Tjio	Chief, Section on Cytogenetics	LEP	NIAMDD
OTHER:	B. J. White	Medical Officer	LEP	NIAMDD
	E. S. Raveche	Research Chemist	LEP	NIAMDD

COOPERATING UNITS (if any)

U. Reinke	Harvard Medical School, Boston
C. Brecher	Univ. Calif., Berkeley, California
A. Steinberg	(AR:NIAMDD)

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Application of differential staining techniques and continued improvement of established methods are basic to all of the projects conducted in the laboratory.

2. Human cytogenetic studies of patients with congenital and developmental disorders as well as studies of mutagen-induced chromosomal abnormalities are continuing.

3. Murine cytogenetic studies include evaluation of mitotic and meiotic chromosomes and karytypic evaluation of cells in hematopoetic tissue regeneration experiments. These are part of projects utilizing the mouse as a model for human diseases.

PHS-6040

Project Description:

Cytogenetics of Hemopoetic Disorders:

Sister chromatid exchange frequencies were used as a criterium for damage and repair processes occurring with leukemia or preleukemia patients. In addition, sister chromatid exchange abnormalities of the hematopoietic system induced by benzene nad related compounds are being studied. Collaborative work with Dr. Germain, Univ. Claude-Bernard, Lyon, France.

Experiments on the Kinetics of Transfused stem Cell in Normal Mouse:

For the past two years in collaboration with Dr. G. Brecher, University of California, we have explored the fate of pluripotential hemopoietic stem cells after transfusion into normal (non-irradiated) mice. In contrast, to the ready replacement of irradiated marrow by transfusion of congenic marrow cells, proliferation of transfused stem cells is very limited in normal marrow. In a study previously reported we suggested that permanent colonization of normal stem cells in normal recipients requires triggering of the stem cells into cycle and possible, in addition, the emptying of special proliferative sites of stem cells. In more recent studies, we found, however, that the existence of such special "niches" of pluripotential stem cells cannot be maintained, because in previously irradiated animals restored to hematologically normal multiple transfusion of bone marrow result in repeated "takes" without any evidence of a restricted number of proliferative sites of niches. Rather it appears that pluripotential stem cells which are normally shed continuously into the peripheral blood cannot reenter the marrow, except after prior irradiation or other modes of severe myelosuppression.

We are now exploring the effect of local irradiation on the control of the reentry of stem cells into the marrow. The results may be of potential clinical significance because local irradiation may be a practical means of replacing functionally deficient marrow in compatible donor-recipient combinations in which at present the risks of severe general myelsuppression (appropriately used for marrow transfusion in leukemia) is unacceptable.

In the course of these studies we noted that stem cells from male donors can be induced to proliferate in female congenic or isogenic recipients, while marrow from female donors will not proliferate in male recipients. The phenomenon occurred in each of several strains tested. The observations cannot be readily explained on immunological grounds, because X chromosome associated antigens are rare and have not been reported as a source of transplant incompatibility in non-immunized hosts. The possibility of female stem cells undergoing "allergic death" in a predominantly foreign male, hence recognizably foreign environment, is being explored.

Clinical Cytogenetic Studies:

For the karyotypic diagnosis of patients referred to our laboratory

routine karyotyping is performed. In addition, because of the undetermined nature of many of the patient disorders, more complicated cytogenetic techniques are involved. Giemsa and quinacrine banding is employed. Additional procedures such as centromeric banding, sister chromatid exchange or late replication studies are sometimes performed.

Murine Cytogenic Studies:

This study is a collaborative project with Dr. Elizabeth Raveche and Dr. Alfred Steinberg, NIAMDD. We have found and previously reported that the autoimmune murine strain NZB develops hyperdiploidy in the spleen. This is inherited as a recessive trait in crosses with the non-autoimmune strain DBA/Z. We have been investigating this further in other strains to determine if the genetic background or immunological status determines the development of hyperdiploidy. To date, in all of the crosses we have found the hyperdiploidy in the spleen is a recessive trait and appears to be an end-result of the NZB autoimmune disease.

Flow Cytometric Analysis:

Using the ability of fluorescent dyes to intercalate DNA and produce a fluorescence emission, we are studying the cell cycle kinetics of normal mice and humans based on DNA changes which the cell undergoes.

Murine Thymic Receptors:

In collaborative work with Dr. Elizabeth Raveche, we have been investigating sex hormone receptors in the thymus of mice. We have detected thymic androgen and estrogen receptors.

Publications:

Brecher, G., Tjio, J. H., Haley, J. E., Narla, J., Beal, S. L.: Transplantation of murine bone marrow without prior host irradiation. Blood Cells 5: 237-246 (1979).

Raveche, E. S., Tjio, J. H., Steinberg, A. D.: Genetic studies in NZB mice. IV. The effect of sex hormones on the spontaneous production of anti-T cells autoantibodies. Arthritis Rheum. 23: 48-56, 1980.

Raveche, E. S., Tjio, J. H., Boegel, W. A., Steinberg, A.D.: Studies of the effects of sex hormones on autosomal and X-linked genetic control of induced and spontaneous antibody production. Arthritis Rheum. 22: 1177-1187, 1979.

White, B. J., Crandall, C., Flier, J., Raveche, E., Tjio, J. H.: Cytogenetic studies of a male with sporadic intestinal lymphangiectasia: 45, X/46, XY Mosaicsim with pseudo- and hyperdiploid subpopulations in cultured tissues. Amer. J. of Med. Genetics 3: 341-352, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,009-13 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cytogenetic Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. J. White	Research Medical Officer	LEP	NIAMDD
OTHER:	J.-H. Tjio	Chief, Section on Cytogenetics	LEP	NIAMDD

COOPERATING UNITS (if any)
J. Goudsmit, CNS, NINCDS; S. Rosen, CE, NIAMDD; E. Bersu, Dept. Anatomy, Univ. of Wisconsin, Madison; W. Strober, MET, NCI; Inter-Institute Genetics Unit, NIH

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Section on Cytogenetics

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Studies of patients and their families with certain genetic disorders due to chromosomal abnormalities or single gene defects. Techniques used include newly-developed chromosomal banding and differential staining, analysis of genetic markers such as enzymes or HLA within pedigrees, and dermatoglyphic analysis. Findings are correlated with phenotypic abnormalities and may be utilized for genetic counseling.

2) Karyotypic evaluation of tissues from laboratory animals which are models of different human genetic disorders, primarily primate and murine. Relationship of cytogenetic variation to defects associated with certain diseases is stressed. Effects of gross chromosomal abnormality upon reproductive fitness and growth and development are studied.

PHS-6040

Project Description:

Studies of patients with genetic disorders:

These investigations of NIH patients with chromosomal abnormalities or single gene disorders are now largely carried out by this investigator in conjunction with the NIH Inter-Institute Genetics Unit. Our own studies emphasize the determination of the effects of chromosomal defects upon growth and development, particularly gonadal development and function. For these studies, techniques used for differential staining of chromosomes include Giemsa-, quinacrine-, centromeric-, and BUdR-Hoechst 3325-banding. In certain cases, the Yunis technique for prometaphase banding is utilized, since it produces more fully-extended chromosomes with delineation of greater numbers of bands. The latter method has been frequently utilized this past year to determine if small chromosomal lesions can be visualized in syndromes associated with severe phenotypic abnormality or impaired mental function.

We are now concluding a study of aneuploidy, frequency of chromosomal aberrations, and chromosome 6 markers in patients with Alzheimer disease, their families, and matched controls in collaboration with Dr. J. Goudsmit (CNS, NINCDS). Our main goals in the study were (1) Either confirm or deny a report from another laboratory claiming increased aneuploidy in Alzheimer disease which might then serve as a useful "marker" for genetic counseling, (2) Determine with prometaphase banding if there is a visible chromosomal lesion associated with the disease which might prove of value in detection before development of clinical abnormalities, and (3) Analyze segregation of HLA and other linked markers in familial Alzheimer's to determine if linkage with the disease is present. Analysis of all specimens is now completed, and the preliminary results are as follows: (1) We were unable to detect increased aneuploidy or other aberrations in affected persons, (2) Chromosomal lesions were not evident in affected persons, and (3) Alzheimer disease does not appear to be genetically linked to HLA. A manuscript concerning the clinical and genetic analysis has been completed, and one concerning the cytogenetic analysis is in preparation.

In collaboration with Dr. S. Rosen (CE, NIAMDD), a genetic study of hypogonadotropic hypogonadism with anosmia (the Kallman syndrome) has been completed. This survey of 18 families indicated that genetic heterogeneity of the syndrome may exist. Previous investigators have reported families in which transmission of the disorder appears to the X-linked, but inheritance in our largest family was most likely autosomal recessive or multifactorial. Transmission compatible with autosomal dominance was observed in several of our other families. There were no chromosomal abnormalities or unique dermatoglyphic traits in affected persons. An association of the syndrome with diabetes mellitus and obesity was observed, but its signficance remains to be determined. A report concerning several male patients proven to father normal children is in press, a manuscript concerning the genetic analysis has been completed, and a report describing associated anomalies and endocrine studies is in preparation.

We plan to continue chromosomal studies of fibroblasts from patients with intestinal lymphangiectasia with Dr. W. Strober (MET, NCI). Studies of four patients have been completed, including one in which transmission was autosomal dominant. Clones with specific autosomal trisomy were observed in fibroblast cultures form two sporadic cases. In the next several months, cultures from a fifth patient with a dominantly-inherited form and a proven cellular defect in folate uptake will be initiated. This is a long-term study which we plan to continue as more patients become available, hoping to learn more about the relationship between the chromosomal abnormalities, the cellular defect, and natural history of the disease.

Cytogenetic studies of selected neurofibromatosis patients being evaluated by the Neurofibromatosis Study Group have heen initiated. Detection of chromosomal variants segregating with this autosomal dominant disease might prove valuable for genetic counseling; other investigators in the group are conducting clinical evaluations of patients and unaffected relatives, and segregation of various genetic markers will be analyzed. Cytogenetic and dermatoglyphic studies of patients with other genetic syndromes such as Pseudohypoparathyroidism are continuing. Since the question of whether the latter disease and 45,X Turner syndrome have similar dermatoglyphic features has not been resolved (White, B. & Marx, S., Clin. Genet. 13: 359, 1978), we plan to study additional patients this coming year.

Karyotypic Studies of Laboratory Animals:

Studies of murine translocation trisomy are being continued in collaboration with Dr. Edward Bersu (Madison, Wisconsin). Previous investigations emphasized description of morphological abnormalities associated with trisomy 19, which included developmental retardation and specific anomalies such as cleft palate. Present experiments are aimed at determining effects of trisomy upon parameters such as cell cycle kinetics and cellular size and number. Flow cytometry, Coulter cell sizing and counting, and BUdR-Hoechst replication analysis in vitro will be utilized to make these determinations. Other investigations of both human and murine trisomic cells have given conflicting results concerning effects of trisomy at the cellular level, and hope to provide a more definitive description of this problem.

Chromosomal studies of primates with genetic disorders also continue. Recently, we have karyotyped chimpanzees suspected to have the same androgen-binding defect as the X-linked testicular feminization syndrome in humans. These collaborative studies have resulted in the first demonstration of this problem in primates; a report of these findings is planned.

Publications:

White, B. J., Crandall, C., Flier, J. S., Raveche, E. S. and Tjio, J.-H.: Cytogenetic studies of a male with sporadic intestinal lymphangiectasia: 45, X/46,XY mosaicism with pseudo- and hyperdiploid subpopulations in

cultured tissues. Amer. J. Med. Genet. 3: 341, 1979.

Curtis, W. R. S., White, B. J., Lucky, A. W., Roche-bender, N., Knab, D. R. and Johnsonbaugh, R. E.: Gonadal dygenesis with mosaicism and non-fluorescent Y chromosome: Report of two cases with correlation of clinical, pathologic, and cytogenetic findings. Amer. J. Obstet. & Gynecol. 136: 639, 1980.

Goudsmit, J., White, B. J., Weitkamp, L. R., Keats, B. J. B, Morrow, C. H. and Gajdusek, D. C.: Familial Alzheimer's disease in two kindreds of the same geographic and ethnic origin: A clinical and genetic study. J. Neurol. Sci. (Oxford) (in press).

Rogol, A. D., Mittal, K., White, B. J., McGinniss, M. H., Lieblich, J. M. and Rosen, S. W.: HLA-compatible paternity in two "fertile eunuchs" with congenital hypogonadotropic hypogonadism and anosmia (the Kallman syndrome). J. Clin. Endocr. Metab. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,010-04 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Function of DNA virus genomes in animal cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barrie J. Carter	Research Biologist	LEP	NIAMDD
OTHERS:	Catherine A. Laughlin	Senior Staff Fellow	LEP	NIAMDD
	Maureen W. Myers	Staff Fellow	LEP	NIAMDD
	Francis T. Jay	Visiting Associate	LEP	NIAMDD
	Carol J. Marcus	Senior Staff Fellow	LEP	NIAMDD

COOPERATING UNITS (if any)
T. Shenk and N. Jones, Connecticut; H. Westphal, and W. Richardson, NICHD
L. M. de la Maza, University of California, Irvine

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SECTION
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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.7	3.7	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are employing DNA viruses as molecular probes to study genome expression in human cells. We are studying intensively the structure and function of adeno-associated virus (AAV) since this virus has only a small genome. We are studying also adenovirus which is a more complex genome. Adenovirus is the helper virus for AAV multiplication. This helper relationship is being analysed. Also both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus this causes malignant transformation of the cell. AAV inhibits this transformation and also inhibits Ad12 oncogenesis in newborn animals. Thus AAV inhibits tumor induction. The mechanism of this inhibition of tumor induction is being studied at the molecular level in both cell culture and in animal experiments.

PHS-6040
(Rev. 10-76)

Project Description:

Adeno-associated virus (AAV) is a defective parvovirus. Because of its small DNA genome and unusual mode of segregation of complementary DNA strands into separate virions, AAV offers a unique situation amenable to biochemical analysis. AAV synthesizes only a few mRNA species (for its coat protein). Thus, the AAV genome seems to consist of a single unique gene sequence enclosed by DNA regions containing reiterated sequences. We are studying AAV as a model system for eykaryotic gene expression. The main objectives of this study are: to analyse the structure of AAV DNA and obtain an accurate transcription map; to study the control of synthesis, post transcriptional processing and modification and transport and degradation of AAV RNA, to analyse replication of AAV DNA; and to study interactions between AAV and its helper adenovirus in both permissive and non-permissive cells. In order to analyse the interaction between AAV and adenovirus we require additional information directly relating to adenovirus function in infected cells. We have therefore initiated studies on various parameters of the adenovirus growth cycle.

Major findings since the last report are:

Molecular structure of AAV DNA variants:

When lysates of human cells, infected jointly with the defective parvovirus, adeno-associated virus (AAV) and a helper adenovirus, are banded to equilibrium in CsCl buoyant density gradients, virus particles of various densities are obtained. Infectious AAV particles mainly band at a density of 1.41g/cm^3 with a minor component at 1.45g/cm^3. Non-infectious AAV particles band at densities between 1.41 and 1.32g/cm^3. We have analysed, by mapping with site-specific endodeoxyribonucleases, the molecular structure of the variant AAV DNA molecules obtained from these light-density particles. The size of variant DNA molecules ranged from 100% to 3% of genome length. In general, the variant DNAs are deleted for internal regions but retain the genome termini. Some of the variant DNAs appear to be cross-linked, spontaneously-renaturing molecules having structures analogous to replicating forms of AAV DNA. This work shows that most of the variant molecules arise by a recombination mechanism rather than by aberrations in replication involving a strand switch by the polymerase.

Structure of heavy and light infectious AAV particles and AAV assembly:

KB cells coinfected with adenovirus and adeno-associated virus (AAV) yielded two kinds of infectious AAV particles that banded in CsCl at densities of 1.45 and 1.41 g/cm^2, respectively. The 1.45 band was found to be composed of a heterogeneous group of viral particles that could be subfractionated by velocity sedimentation. The main component from this band had a smaller S value (109) than the main component from the 1.41 band (115), although both had the same DNA protein ratio and

the same density in metrizamide gradients. Continuous-label experiments showed that early after infection, both components (1.45 and 1.41) were generated in the same amounts, but this was followed by a relative increase in the proportion of the 1.41 component over the 1.45 particles. Pulse-chase analysis failed to demonstrate a precursor-product relationship between these two bands. The slower-sedimenting components from the 1.45 band were unstable in Cs Cl and were present in a greater proportion early after infection. These particles contained DNA that was enriched for the terminal sequences of the AAV genomes and was accessible to digestion with micrococcal nuclease.

The assembly of the defective parvovirus, adeno-associated (AAV), was studied by labeling DNA and protein precursors with [^{3}H]thymidine or [^{35}S]methionine, respectively. Virus particles were extracted from cell lysates and characterized by banding to equilibrium in CsCl gradients and by velocity sedimentation in neutral sucrose. Radioactive pulse-chase labeling experiments showed that newly synthesized AAV proteins are rapidly assembled into empty capsids which in turn serve as precursors to stable, mature (full) particles that contain either a standard or deleted (defective-interfering) virus genome. While association of the newly synthesized progeny DNA strands with empty capsids appears to be rapid, maturation to form a stable form particle apparently is a much slower process requiring several hours. A new component, having the properties of an AAV genome associated with the empty capsid in a DNase-sensitive conformation (in contrast to the complete DNase resistance of DNA in mature particles), also exhibited pulse-chase kinetics consistent with a role as an assembly intermediate. L-Canavanine inhibited production of empty capsids and thus inhibited assembly of full particles. Pretreatment of infected cultures with hydroxyurea inhibited appearance of full particles and caused accumulation of empty capsids consistent with their precursor role. Addition of hydroxyurea immediately after a pulse label showed that maturation of AAV particles appears to occur in the absence of concomitant DNA replication. These experiments are consistent with a model of AAV assembly in which progeny DNA strands are packaged into preformed capsids.

AAV RNA Transcription:

We have continued our studies on transcription of AAV in RNA in human KB cells. We have analysed the structure of cytoplasmic RNA species transcribed from DNA of adenovirus-associated virus, a defective parvovirus. The RNA was hydridized with minus strand template DNA and visualized in the electron microscope. Alternately, the DNA-RNA duplex molecules were digested with nuclease S1 or Escherichia coli exonuclease VII and analyzed by agarose gel electrophoresis. A set of RNA species was observed with 5' termini at map positions 5, 13, 19, or 39 and a 3' terminus and poly(A) tail at position 96 (one map unit is equivalent to 1% of genome length). Most of these RNAs are spliced and lack sequences approximately between positions 40 and 49. Some RNA preparations also contained unspliced molecules with 5' and 3' termini at positions similar to those in the spliced RNA.

Z01 AM 21,010-04 LEP

We have employed Ad mutants to determine which genes may be required for the AAV helper function. Two mutants of Ad5, ts125 and ts107, with temperature-sensitive lesions in the E72 DNA binding protein coded by the Ad early region 2, were deficient for AAV helper functions at the non-permissive temperature (40°). In contrast, Ad5ts149 with a temperature-sensitive lesion in the Ad early region 5 was an efficient helper of AAV at the non-permissive temperature. In KB cells, with the Ad5ts125 or Ad5ts107 mutant as the helper, the accumulation of AAV capsid proteins and AAV particles was decreased by only two logs whereas AAV DNA synthesis was decreased only several fold. Cytoplasmic, poly(A)-containing AAV RNA comprises a set of overlapping, spliced RNAs having different 5' start points. With the ts125 helper at 40°, there was a decreased accumulation of some but not all of these AAV RNAs. The Ad5 E72 protein may have an effect on transcription or more likely post-transcriptional processing of AAV RNA. These observations suggest additional pleitropic effects of the multifunctional E72 protein and suggest further similarities in the actions of E72 and the SV40 T-antigen.

Publications:

Jay, F. T., de la Maza, L. M. and Carter, B. J.: Parvovirus RNA transcripts containing sequences not present in mature RNA. A method for isolation of putative mRNA precursor sequences. Proc. Natl. Acad. Sci. USA. 76: 625-629, 1979.

Handa, H., and Carter, B. J.: Adeno-associated virus DNA replication complexes in Herpes Simplex virus or adenovirus infected cells. J. Biol. Chem. 254: 6603-6610, 1979.

Laughlin, C. A., Westphal, H. and Carter, B. J.: Spliced adenovirus-associated virus RNA. Proc. Natl. Acad. Sci. USA 76: 5567-5571, 1979.

Richardson, W. D., Carter, B. J. and Westphal, H.: Vero cells injected with adenovirus type 2 mRNA produce authentic viral polypeptide patterns: Early mRNA promotes growth of adenovirus-associated virus. Proc. Natl. Acad. Sci. USA. 77: 931-935, 1980.

de la Maza, L. M., and Carter, B. J.: Heavy and light particles of adeno-associated virus. J. Virol. 33: 1129-1137, 1980.

Myers, M. W., and Carter, B. J.: Assembly of adeno-associated virus. Virology. 102: 71-82, 1980.

de la Maza, L. M., and Carter, B. J.: Molecular structure of adeno-associated virus variant DNA. J. Biol. Chem. 255: 3194-3203, 1980.

Myers, M. W., Laughlin, C. A., Jay, F. T., and Carter, B. J. The adenovirus helper function for growth of adeno-associated virus: The effect of temperature sensitive mutations in adenovirus early gene region 2. J. Virol. 35: in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,012-03 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cytogenetics and Immunogenetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	J. H. Tjio	Chief, Section on Cytogenetics	LEP	NIAMDD

COOPERATING UNITS (if any)

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O. Alabaster, NCI
R. Viergersky, Walter Reed, Wash., D.C.
D. Shreffler, Washington University School of Medicine, St. Louis, Mo.
J. Taurog, NIAMDD

LAB/BRANCH
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Section on Cytogenetics

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Application of differential staining techniques for chromosome analysis.

2. Studies of the inheritance of several autoimmune immunological traits including antibodies cytotoxic for T lymphocytes and reactive with single stranded DNA.

3. Studies of inheritance of hyperdiploidy.

4. Analysis of sex hormone modulation of immune responses.

5. Use of flow cytometry in the analysis of aneuploid cells.

6. Sex hormone receptors in murine thymus.

Project Description:

Murine Immunogenetic Studies:

NZB mice develope an autoimmune disease characterized by antibodies to T cells, erythrocytes, and single stranded DNA (ssDNA), and hyperdiploidy. It has been suggested that multiple genes are responsible for the disease of these mice; however, there is still considerable controversy regarding the number of genes responsible for autoimmunity in NZB mice, as well as the interrelationships of different autoimmune traits. We have been investigating the genetic basis for the production of autoantibodies, as well as the role of sex hormones in their expression. In this study, we analyzed recombinant inbred (RI) mice at one year of age from lines between F_{12} and F_{18} generations made from NZB X DBA/2, NZB X CBA, and NZB X ALN mice. We found individual RI lines expressing separately only anti-ssDNA antibodies; anti-T cell antibodies; anti-erythrocyte antibodies; and hyperdiploidy. Moreoever, some lines showed suppression of anti-T cell anti-ssDNA antibodies by androgens, whereas others manifested the androgen resistance seen in NZB mice. In addition, in some of the RI lines, the female mice, like parental NZB females, abnormally produced sex limited serum protein (Slp) usually found only in males. In RI mice, NTA production was not linked to either androgen insensitivity or to abnormal Slp production. Of particular note, anti-ssDNA and anti-erythrocyte antibodies occurred in the absence of anti-T cell antibodies.

From the results of these studies, it appears that separate genes are associated with separate autoimmune phenomena of NZB mice. Nevertheless, it is possible that their expression could require a fundamental immune regulatory defect.

Murine Cytogenetic Studies:

Experiments in collaboration with Drs. A. Steinberg, J. H. Tjio and Joel Taurog (NIAMDD) to determine the effects of genetic background on the expression of hyperdiploidy in various crosses with NZB mice are currently underway. Hyperdiploidy is one of the end results of autoimmunity. The appearance of hyperdiploid cells was studied in (NZB · CBA/N) congenic mice which were greater than 90% NZB-like except for the presence of the defective CBA/N X chromosome. The hyperdiploid cells were absent as was the autoimmune disease normally found in NZB mice which suggests that the NZB hyperdiploid cell may be fundamental in the development of autoimmunity.

Flow Cytometric Studies:

In order to study the cell types involved in the hyperdiploidy characteristic of NZB mice, flow cytometric techniques which measure the DNA content of individual cells were employed. The aneuploid cells present in NZB mice were clonally derived of a larger size than normal cells. They could not be removed by anti-Thy 1 plus complement nor were they

present in the nylon-wool column non-adherent fraction, both of which are characteristic of T cells. By further cytotoxic analysis, the aneuploid cells were found not to express Ly-5 nor NK surface antigens, found on natural killer cells. The aneuploid cells had increased quantities of cell surface H-2 antigen which is to be predicted because of the one or more extra copies of chromosome 17, which carries the H-2 complex. The aneuploid cells found in the spleen of older NZB mice were derived from bone-marrow stem cells. A proportion of these cells had surface Ia and membrane Ig, both of which are characteristic of B cells. In addition, neonatally thymectomized and lethally radiated NZB X DBA/2 recipients of anti-Thy-1 treated young NZB bone marrow cells ultimately developed aneuploid spleen cells.

Immune Androgen Insensitivity:

For both autoantibodies against T cells and those reactive with single-stranded DNA, males usually have a lower response than females. The females can be suppressed by administration of testosterone. The higher responses in females and suppression by testosterone were true for all mice studied except NZB mice, the murine model for autoimmune disease. NZB mice appear to have an insensitivity to the suppressive effects of testosterone. The cell population on which testosterone ordinarily acts may be absent. We have investigated this further by studying androgen receptors in NZB thymus.

In addition, in work with Dr. Donald Shreffler, Washington University, St. Louis, we have studied a serum protein (Slp) which is dependent on testosterone for its appearance. NZB females and castrated males are abnormal and produce Slp. This is not related to any other immunological finding and seems to be a separate phenomenon.

Hormone Receptors:

In collaboration with Dr. R. Viergersky, Walter Reed Army Medical Center, we are studying sex hormone receptors in the thymus. Since sex hormones profoundly effect the immune response, we are searching for the mechanism by which hormones mediate this action. We have identified a β-estradiol specific cytosol receptor in the murine thymus. We have also identified a thymic androgen receptor in both females and males with a $k_d = 2 \times 10^{-9}$, $B_{max} = 30$ pm and an approximate molecular weight of 400,000. This receptor translocated into the nucleus in all strains studied except NZB males. This failure to detect nuclear translocation of the thymic androgen receptor may in part, be responsible for NZB immune androgen insensitivity.

Thymus Transplants:

Because the thymus has sex hormone receptors, we are initiating experiments involving neonatal thymectomies followed by opposite sex thymus grafts. By this technique, we hope to further investigate the postulated immune androgen insensitivity of NZB mice and the role of the

thymus in sex differences. Thymic grafts did not alter sex pattern of slp production. We are currently investigating tolerance to bovine gamma globulin (B66).

Publications:

Raveche, E. S., Tjio, J. H., Steinberg, A. D.: Studies of the effects of sex hormones on autosomal and X-linked genetic control of induced and spontaneous antibody production. Arthritis and Rheumatism. 22: 1177-1187, 1979.

Raveche, E. S. and Steinberg, A. D.: Lymphocytes and lymphocyte function in SLE. In Clinics in Hematology ed. H. Fudenberg 16: 344-370, 1979.

Steinberg, A. D., Melez, K. A., Raveche, E. S., Reeves, J. P., Boegel, W. A., Smathers, P. A., Taurog, J. D., Weinlein, L. M., Duvic, M.: Approach to the study of the role of sex hormones in autoimmunity. Arthritis and Rheumatism. 22: 1170-1176, 1979.

Raveche, E. S., Tjio, J. H., Steinberg, A. D.: Genetic studies in NZB mice. IV. The effect of sex hormones on the spontaneous production of anti-T cell autoantibodies. Arthritis and Rheumatism. 23: 48-56, 1980.

Huston, D. P., Raveche, E. S., and Steinberg, A. D.; Preferential lysis of undifferentiated thymocytes by natural thymocytotoxic antibodies from New Zealand black mice. J. Immunology. 124: 1635-1641, 1980.

Steinberg, A. D., Melez, K. A., Klassen, L. W., Williams, G. W., Reinersten, J. L., Reeves, J. P., Boegel, W., Smathers, P. A., Gerber, N. L., Duvic, M., and Raveche, E. S.: Newer approaches to the treatment of systemic lupus through study of New Zealand Mice. Proc. International Meeting on SLE, Kyoto, Japan, Nov. 1978. Edited by Homma and Fukase, Tokyo University, Japan Press, 1980.

Steinberg, A. D., Roths, J. B., Murphy, E. D., Raveche, E. S.: Effects of thymectomy or androgen administration upon the autoimmune disease of MRL/Mp-lpr/lpr mice. J. Immunol. (in press).

White, B. J., Crandall C., Flier, J. S., Raveche, E., Tjio, J. H.: Cytogenetic studies of a male with sporadic intestinal lymphangiectasia. Am. J. of Medical Genetics. 3: 341-352, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,013-03 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Electron Microscopic Investigations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Howard A. Bladen	Research Biologist	LEP	NIAMDD
OTHER:	Jacqueline Henson	Histopathologist	LEP	NIAMDD

COOPERATING UNITS (if any)
T. Chai, LBM, NIAMDD; R. Maheshwari, LEP, NIAMDD; E. Grollman, LPP, NIAMDD; B. Rosset, LEB, NIAMDD; G. Lee, LBP, NIAMDD; D. Gill, LBP, NIAMDD; R. Goldman, LBP, NIAMDD

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During the last year, this unit has been involved in studies with several investigators. As in the past some have been on a continuing basis while others have been short term and involved essentially in screening of samples.

PHS-6040

Project description:

In collaboration with Dr. D. Gill, we have performed electron microscopy studies of synaptosomal lysates in order to determine the relative content of plasma membranes, mitochondria, endoplasmic reticulum and neurotransmitter storage vesicles. This has involved various techniques in preparing fractions, representative sampling and comparative identification of organelles in both the intact synoptosome and different fractions isolated by density gradients from synaptosomes. The identification of plasma membrane vesicles in those fractions containing ATP-dependent calcium transport and sodium - calcium exchange has recently resulted in the localization of such calcium transport mechanisms in the plasma membranes. This evidence has been submitted for publication in J. Biological Chemistry.

Dr. Grollman requested that we investigate the purity and identification of preparations used for biochemical studies on the effects of electrochemical gradients induced by various ligands on specific tissue. In particular, we have observed thyroid follicular structure as well as intracellular vesicles and villi in cells from thyroid cells in tissue culture preparations. These morphological findings correlate well with the functional state and the transport properties of the cells. In addition, synoptosomal elements and mitochondria were identified in preparations of mouse brain cells used for studies on the effect of tetanus toxin and the uptake of calcium. Membrane vesicles from fibroblast were screened by electron microscopy to determine their purity for further biochemical studies.

Dr. Maheshwari has reported data which shows a significant reduction in glycoprotein and membrane protein of VSV particles released from IF-treated cells. Additional evidence concerning the deficiency of glycoprotein in IF-treated cells was obtained from E. M. studies. VSV particles released from IF-treated cells has relatively less surface structure termed spikes than those VSV particles released from non IF-treated cells. The reduction in the number of spikes present on the VSV particle appeared to be dependent on the concentration of IF used to treat the cells.

Dr. G. Lee is studying a hormone-dependent functional thyroid cell strain and a non-functional thyroid cell strain from rat. These are being used in a comparative study of their biological and biochemical properties, i.e., iodine uptake, thyroglobulin biosynthesis and degradation and c-AMP levels. Both thyroid cell strains are interferson sensitive against virus infection. Electron microscopy has been used to screen various cell preparations in order to assure sample homogenity for biochemical studies.

In addition to the above mentioned studies, we have performed services for Dr. Rousset (Microtubules), Dr. Chai (Bacteria), and Dr. Goldman (Bacteria). These studies were of short duration and did not require and continuous effort.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,014-03 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Interferon Inhibition of Viruse(s) Which Bud From Plasma Membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Radha Maheshwari	Visiting Fellow	LEP	NIAMDD
OTHER:	Robert M. Friedman	Chief	LEP	NIAMDD

COOPERATING UNITS (if any)
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A 200-fold inhibition in the titer of infectious vesicular stomatitis virus (VSV) was achieved in cultures of L-cells treated with 30 reference units/ml of interferon (IF); however virus particle production, as measured by VSV particle-associated RNA, nucleocapsid (N) protein, or transcriptase activity, was inhibited by a maximum of 10-fold by this concentration of Interferon. A significant reduction was seen in the glycoprotein (G) and matrix protein (M) content of VSV from interferon-treated cells. Electron-microscopic studies also support the deficiency of glycoprotein in VSV released from interferon or tunicamycin-treated cells. The incorporation of N-acetylglucosamine into glycolipids with the properties of dolichol derivatives is lower in membranes prepared from interferon-treated cells as compared to the untreated cells. These results suggest that interferon inhibits the glycosylation of a viral protein in this system.

PHS-6040

Project Description:

As much as 200-fold inhibition in the titer of infectious vesicular stomatitis Virus (VSV) was achieved in cultures of L-cells treated with 30 reference units/ml of interferon (IF); however virus particle production, as measured by VSV particle-associated RNA, nucleocapsid (N) protein, or transcriptase activity, was inhibited by a maximum of 10-fold by this concentration of Interferon. A significant reduction was seen in the glycoprotein (G) and matrix protein (M) content of VSV from interferon-treated cells. Evidence supporting the deficiency of glycoprotein in VSV released from interferon-treated cells was derived from electron-microscopic studies. Under conditions where glycoprotein spikes or projections were clearly detectable on the surface of VSV released from cells not treated with Interferon, very few spikes were observed on VSV released from interferon-treated cells. These results suggested that interferon-treated cells produced VSV particles with low infectivity and that this low infectivity may be related to the reduced amount of glycoprotein and membrane protein incorporated into such particles. Only 42S viral RNA was detected in the virus particles produced by interferon treated cells; these particles did not interfere with the growth of wild type VSV in BHK or L-cells. The finding suggested that these particles are not defective-interfering forms of VSV. The results in many respects resemble those previously reported in interferon-treated cells infected with murine leukemia viruses.

Previous investigations on the ultrastructure of VSV had demonstrated the presence of spikes on the surface of wild-type virus particles when examined by negative staining techniques. The identity of the glycoprotein of VSV as the spike protein has been confirmed by treatment of these particles with proteases such as pronase, which digested the surface proteins without rupturing the lipid bilayer; this resulted in the removal of the spikes with the concomitant producion of non-infectious viral particles. The production of non-infectious virus particles morphologically and biochemically differing from wild type particles has been reported in group V temperature sensitive (ts) mutants of VSV grown at non-permissive temperatures and also with some mutants of Rous Sarcoma Virus. In several instances the production of non-infectious virus was associated with the absence of major glycoproteins. These data suggest that at least some of the reduced infectivity of the VSV particles released from interferon-treated L-cells may well be related to the reduced amount of glycoprotein incorporated into such particles.

It is possible that interferon-induced changes in the cell plasma membrane may be the basis for the alteration in infectivity of both VSV the murine leukemia virus produced after treatment with interferon because the morphogenesis of these viruses involves budding from the cell surface.

We have compared the effect of low doses os interferon with that of tunicamycin (a glucosamine containing an antibiotic, which inhibits the lipid intermediate pathway by blocking the transfer of N-acetylglucosamine

1-phosphate from UDP-N-acetyl glucosamine to dolichyl monophosphate) on various parameters of VSV released from L-cells. There was a 200-fold inhibition of infectious VSV particles released from cells treated with interferon; tunicamycin (TM) inhibited the production infectious VSV by 10-80 fold. Earlier we showed that VSV released from cells treated with interferon was deficient in G and M protein. With TM (1 μg/ml) treatment we obtained complete inhibition of G protein glycosylation; however M protein was also inhibited which suggested that there is a linkage between G and M protein, since both of these proteins are membrane associated. The VSV particles released from cells treated with interferon or tunicamycin were morphologically similar in lacking glycoprotein spikes.

In vitro studies have shown that the synthesis of N-acetylglucosamyl lipids with properties of dolichol derivatives, that serves as precursors for the dolichol-bound oligosaccharide lipid intermediates in protein glycosylation was lower by membrane preparations from interferon-treated cells than similar membrane preparations from untreated cells. Similar results were obtained from membrane preparations treated with interferon and infected with VSV. This effect could be partially responsible for the results which we obtained with VSV released from L-cells treated with interferon. Such virions were selectively deficient in G and M protein. It might also explain results on Moloney murine leukemia virus (MMLV) from interferon-treated TB cells, where virus with markedly decreased infectivity was producted.

Publications:

Maheshwari, R. K. and Friedman, R. M.: Production of vesicular stomatitis virus with low infectivity by interferon-treated cells. J. Gen. Virol. 44: 261-264, 1979.

Maheshwari, R. K., Jay, F. T. and Friedman, R. M.: Selective inhibition of glycoprotein and membrane protein of vesicular stomatitis virus from interferon treated cells. Science, 207: 540-541, 1980.

Maheshwari, R. K. and Friedman, R. M.: Effect of interferon treatment on vesicular stomatitis virus (VSV): Release of immune paricles with low infectivity. Virology. 101: 399-407, 1980.

Maheshwari, R. K., Demsey, A. E., Mohanty, S. B., and Friedman, R. M.: Interferon-treated cells release vesicular stomatitis virus particles lacking glycoproteins: Correlation with biochemical data. Proc. Natl. Acad. Sci. USA. 77: 284-2287, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,015-03 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Mechanisms of antiviral activity induced by interferon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Francis T. Jay	Visiting Associate	LEP	NIAMDD
Robert M. Friedman	Chief, Laboratory of Experimental Pathology	LEP	NIAMDD

COOPERATING UNITS (if any)
NONE

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/5	1 1/5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have previously shown that interferon (IF) alters the structure of the plasma membrane. Furthermore, both Moloney murine leukemia virus and vesicular stomatitis virus (VSV) produced by IF-treated cells have low infectivity. While the content of the G protein (envelope glycoprotein) in VSV produced in IF-treated cells is reduced, no defect in the net charge or molecular weight or the extent of glycosylation and silialyation of the G protein was observed. We have also studied the biosynthesis of the viral proteins (VPs). Although IF inhibits the synthesis of VPs to a varying extent depending on the dose, this inhibition is not likely to be responsible for the reduced incorporation of G protein into the virions since the intracellular VPs accumulated is in vast excess of that incorporated into the virion. The relative rate and extent of glycosylation of intracellular G protein in the IF-treated cells are not changed. Glycosylation does not appear to be affected by IF. The decreased amount of G protein in the low infectious virion may be due to blocks in the intracellular transport or utilization of G protein in the packaging of the virion on the plasma membrane.

Project Description:

Mechanism of Antiviral Action of Interferon:

We have previously shown that cells treated with interferon (IF) have altered plasma membrane structure, physcially, morphologically and biochemically. Furthermore, both Molony murine leukemia virus (Mo-MuLV) and vesicular stomatitis virus (VSV) produced by IF treated cells have very low infectivity compared to that of the control viruses. Our effort was (i) to analyze the molecular defects in the low infectious virions which may indicate the enzyme systems or cellular processes involved in the antiviral activity (an indirect approach from excellular to intracellular) and (ii) to study the biosynthesis and transport of the viral proteins in an attempt to identify the intracellular biochemical changes associated with the production of the low infectious virion after treatment with IF, (a direct study to identify cellular changes) as means to understand the molecular mechanism of the antiviral activity induced by interferon.

In the structural analyses of the low infectious particles, we have found that the relative amount of the envelope glycoprotein gp 69/71 is significantly lower in the Mo-MuLV produced in the IF treated TB cells. Recently, similar results were also observed in the VSV. Since VSV is a lytic system that switches off host synthesis, further work was done with VSV. Two dimensional gel electrophoresis (2D-gel) analyses of the VSV viral proteins (VPs) suggest that there is no qualitative changes in the VPs due to IF treatment. The extent of glycosylation and the number and distribution of sialiac acid residues in the G proteins of the low infectious virion and control are similar. Although remote, the possibility that the carbohydrate content, glycosylation sites or the peptide backbone in the low infectious virion G protein is different are presently being investigated. Since the G protein has been shown to be essential for the adsorption of the virion to the cell during infection our results propose that the decrease in infectivity is due to the deficiency, and not the quality, of the evelope glycoprotein incorporated into these particles. No other molecular abnormalities have been found in the other viral proteins except that the amount of M protein may be reduced slightly. The decreased amount of G protein in these particles may be due to having a rate limiting step, induced by IF treatment, in one of the following: (i) production of functional mRNA for G protein, (ii) translation of the mRNA, (iii) rate and extent of glycosylation, (iv) transport of G protein to the plasma membrane and (v) utilization of the G protein in the packaging of the virion.

In the in vivo studies, at least the first three possibilities can be eliminated as the rate limiting step that causes the deficiency of G protein in the virion produced in the presence of IF. Although the inhibition, by IF treatment, of the synthesis of G protein is marginally greater than the other viral proteins the amount of intracellular G protein (and other VPs) is in vast excess of the amount used in the packaging of virions even in the IF treated cultures. Pulse chase experiments demonstrated that the stability of the VPs is not altered and that the

relative rates and extent of glycosylation of the G protein is not affected by IF. Furthermore, the number of siliac acid residues added (reported to be important for infection) to the G protein is also not changed over a large range of IF doses where VP synthesis is inhibited up to 1000 times. Glycosylation is therefore, probably not affected by IF at all, although final confirmation must come from the structural analyses of the carbohydrate moiety mentioned above. The reduced amount of G protein incorporated in the virion is probably due to IF-induced block(s) in the intracellular transportation of G protein or the utilization of this protein in the packaging (and budding) of VSV from IF treated cells. Especially the budding process, very little is known mechanistically. We have just begun to study the transport and sites of accumulation of the VPs, especially the envelope glycoprotein G.

Publications:

Jay, G., Jay, F. T., Chang, C., Levine, A. S. and Friedman, R. M.: Induction of SV40-specific tumor rejection by the $Ad2^{+}ND_2$ hybrid virus. J. Gen. Virol. 44: 287-297, 1979.

Jay, F. T., de la Maza, L. M., and Carter, B. J.: Parvovirus RNA transcripts containing sequences not present in mature mRNA: A method for the isolation of putative mRNA precursor sequences. Proc. Natl. Acad. Sci. U.S.A. 76: 625-629, 1979.

Maheshwari, R., Jay, F. T., and Friedman, R. M.: Selective inhibition of the glycoprotein and matrix protein content of vesicular stomatitis virus released by interferon-treated cells. Science. 207: 540-541, 1979.

Myers, M. W., Laughlin, C. A., Jay, F. T., Carter, B. J.: The adenovirus helper function for the growth of adeno-associated virus: The effect of temperature-sensitive mutations in adenovirus early gene region 2. J. Virol. (in press).

Friedman, R. M., Maheshwari, R., Jay, F. T., and Czarniecki, C. W.: Mechanism of interferon inhibition of viruses which bud from the plasma membrane. Ann. New York Acad. Sci. (in press).

Maheshwari, R., Jay, F. T., and Friedman, R. M.: Structural abnormalities in vesicular stomatitis virions released by interferon-treated mouse L cells. Ann. New York Acad. Sci. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 21,016-02 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Purification of Messenger RNA for Human Interferon.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Robert M. Friedman	Chief, Laboratory of Experimental Pathology	LEP	NIAMDD

COOPERATING UNITS (if any)

Michael Hitchcock, NIAMDD
Shelby Berger, NCI
Kathryn Zoon, NIAMDD
Esther Chang, NCI

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SECTION
Section on Chemical Pathology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The messenger RNA is obtained from human Namalva (lymphoblastoid) cells. Partial purification of this mRNA has led to about a 90-fold enrichment in our best preparations. The enriched preparations are transcribed by an RNA tumor virus reverse transcriptase and double stranded DNA is made from this.

PHS-6040

Project Description:

Assay techniques for interferon mRNA, as with other mRNAs, involve translation followed by detection of the protein product. In the system used as little as 0.05 pg of interferon protein can be detected. However no activity is seen after incubation of mRNA fron interferon producing cells in either the wheat germ or reticulocyte lysate cell-free protein synthesizing systems. Significant activity can be found when translation of such mRNA is performed by Xenopus oocytes and this is believed to be due to the ability of this system to carry out post-translational modifications. This assay system however suffers from errors due to the difficulty in dispensing with accuracy the very small injection volumes which the oocyte can tolerate (about 50 nl per oocyte). Therefore we have devised an apparatus to perform this function which automatically controls dispensing of injection volumes in the nanoliter range. Using this device, we are now able to perform assays which are reproducible.

Induction of Namalva cells with Newcastle Disease virus leads to a 50 to 100 fold increase in the content of interferon mRNA. In contrast to the situation found with fibroblast cells stimulated with poly I:C, this message is persistent in Namalva cells subsequent to cessation of its translation, and the extent of its association with polysomes can be correlated with rate at which interferon is made. This feature may explain the inability to perform the "superinduction" in this system, which is dependent in the fibroblast system on the prolonged maintenance of mRNA levels.

RNA from induced Namalva cells prepared by phenol extraction of cytoplasm has been prepared in the presence of nucleoside-vanadyl complexes. Under these conditions it has been shown that intact, translatable mRNA is obtained. These preparations are enriched for interferon mRNA by oligo dT cellulose chromatography and formamide/sucrose density gradient centrifugation. Enriched interferon mRNA preparations have been used as a template for oligo dT primed reverse transcription and the cDNA has been converted to double stranded DNA, and cloned in E. coli. Attempts to select clones containing DNA coding for interferon sequences will be undertaken when a suitable screening technique is devised.

Publications:

Berger, S. L., Hitchcock, M. J. M., Zoon, K. C., Birkenmeir, C. S., Friedman, R. M. and Chang, E. H.: Characterization of interferon messenger RNA synthesis in Namalva cells. J. Biol. Chem. 255: 2955-2961, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,017-01 LEP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Mechanisms of Interferon Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. David A. Epstein	Staff Fellow	LEP	NIAMDD
OTHER:	Dr. Christine Czarniecki	Staff Fellow	LEP	NIAMDD
	Dr. Robert M. Friedman	Lab Chief	LEP	NIAMDD
	Dr. Helmut Jacobsen	Guest Worker	LEP	NIAMDD
	Dr. Amos Panet	Visiting Scientist	LEP	NIAMDD
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COOPERATING UNITS (if any)

NONE

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	4	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this research is to determine the biochemical basis of interferon action. Our work has focused on 2'5'-isooligoadenylic acid synthetase and a protein kinase activity which phosphorylates a 67,000 dalton protein, two enzymatic activities which are induced by interferon and enhanced by some double-stranded ribonucleic acids (dsRNA). The work has been divided into two studies: 1) The relationship of polynucleotide structure to the activation of 67K protein kinase activity and protein synthesis inhibition and 2) the role of oligo A synthetase in the establishment and maintenance of the antiviral effects of interferon.

PHS-6040

Project Description:

Biochemical Mechanisms of Interferon Action:

1) Role of polynucleotide structures on the stimulation of phosphorylation of the 67K dalton protein.

In accord with previous studies, poly I·poly C, a potent inhibitor of the cell-free protein-synthesizing system of interferon-treated L cells, stimulated incorporation of [^{32}P] from (γ-[^{32}P]ATP into the 67K dalton protein, P_1. The double-stranded RNA poly I·poly br^{5}C, which is inactive as an inhibitor of protein sythesis, did not stimulate phosphorylation of P_1 under conditions approximating those of protein synthesis. However, we have found conditions under which poly I·poly br^{5}C was approximately as effective as poly I·poly C in stimulating incorporation of label from (γ-[^{32}P]ATP into 67K dalton protein. Upon transfer of labeled P_1 from these conditions to those compatible with protein synthesis, there was a time-dependent decrease in label in the 67K-dalton protein. This decrease was more rapid in the presence of poly I·poly br^{5}C than in the presence of poly I·poly C. This differential decrease was also observed when [^{32}P]-labeled extracts were diluted into buffer containing 10mM ATP, hexokinase and 1mM glucose, or Escherichia coli alkaline phosphatase. Partial proteolytic digest of P_1 labeled in the absence of double-stranded RNA or in the presence of poly I·poly C or poly I·br^{5}C gave rise to similar peptide patterns. These results suggested that dephosphorylation as well as phosphorylation determined the net incorporation of [^{32}P] into P_1. Moreover, these results suggested the existence of a phosphatase activity that may be inhibited more strongly by poly I·poly C than poly I·poly br^{5}C.

In addition, a survey was made of the action of the polynucleotides on the 67K-dalton kinase activity. In every case but one, it was found that polynucleotides which were previously shown to inhibit in vitro protein synthesis by Torrence and Friedman (J. Biol. Chem. 254: 1259-1267), stimulated phosphorylation of the 67 K dalton protein under in vitro protein synthesis conditions.

2) Role of iso-oligoadenylate synthetase in the maintenance of the anti-viral effect induced by interferon.

In a project carried out in collaboration with Drs. Panet and Czarniecki, the finding that interferon treatment causes a large (ten-fold) reduction of reverse transcriptase activity in cells chronically infected with murine leukemia virus has been confirmed in the mouse 3T3 cell line. Quite unexpectedly, however, interferon is unable to protect these cells from infection by encephalomyocarditis virus (EMCV) even at relatively high doses. These results prompted a study of enzymes induced in cells treated with interferon. Oligoisoadenylate synthetase is induced in these cells at relatively high doses of interferon (greater than 100 units/ml). However, an in vitro translation system from 3T3-cells chronically infected with murine leukemia virus is completely resistant to the inhibitory action of 2', 5'-oligo adenylate. This observation is consistent with the proposed

role of 2'5',-oligo adenylate in the maintenance of the antiviral effect. It is also the first instance of a system which is resistant to oligo adenylate.

Dr. Jacobsen has been studying in greater detail the role of oligo adenylate synthetase in Krebs II cells. He has confirmed the initial observations of Dr. Torrence which showed that this cell-line has high levels of iso-oligo adenylate synthetase and that it is inhibited by 2',5'-oligo adenylate. This cell line is a good host for EMCV and is not protected by interferon from virus infection. The working hypothesis that Krebs II cells have 1)relatively high levels of the phoshodiesterase which degreades 2',5'-oligo adenylate, 2) have relatively low levels of the nuclease activated by 2', 5'-oligo adenylate, or 3) a combination of the two previous possibilities is now being tested.

Conditions other than interferon-treatment have been tested for their ability to induce iso-oligo adenylate synthetase. Levels of this enzyme do not increase in L-cells incubated either during presence of dexamethasone or following its removal. Dimethylsulfoxide has no effect on the levels of this enzyme in Friend erythroleukemia cells. Serum deprivation has no effect on the levels of this enzyme in 3T3 cells.

Publications:

Epstein, D. A., Torrence, P. F. and Friedman, R. M.: Double-stranded RNA inhibits a phosphoprotein phosphatase present in interferon-treated cells. Proc. Natl. Acad. of Sci. 77: 107-111, 1980.

LABORATORY OF CHEMICAL BIOLOGY, NIAMDD
ANNUAL REPORT: OCTOBER 1, 1979 - SEPTEMBER 30, 1980

A major emphasis in the research of the Laboratory of Chemical Biology has been the further study of the kinetic processes and thermodynamic constraints that underlie the unfolding of polypeptide chains into native protein molecules. This phenomenon has been approached both by examination of complex formation between fragments prepared from a number of proteins and by the chemical synthesis of some of these fragments in both natural and analog forms. The understanding of this folding process is directly applicable to other questions being asked such as the nature of the neurophysin-oxytocin or -vasopressin system, the normal and abnormal species of hemoglobin and human interferon.

The convenient organism, paramecium, is under continuing study in connection with the comparative chemistry of the myoglobins from different species, the surface antigens and the nature of cell-cell interaction controlling mating and mediated through the agglutinen.

As a natural corollary of the studies on chemical characterization and intramolecular organization, the basic studies serve as the framework for biologically oriented projects on normal, fetal and sickle hemoglobin, proteins of mammalian sperm and egg coats and the structure-function and binding properties of human interferon.

I. Chemistry, function and three-dimensional conformation of macromolecules

A. The dynamics of protein conformation

(1) A working hypothesis for the procedures of protein folding has been developed, mostly on the basis of studies of fragment complementation. The model involves four theories. First, protein molecules participate in a dynamic equilibrium between the ordered (native) and the disordered (unfolded) state (this theory has been unequivocally proved using the fragment exchange technique). Second, the disordered state is composed of conformational states which are in equilibrium with each other and which are assumed not to be distributed in all stereochemically possible conformations. A corollary to this theory is that the conformational distribution of the disordered state may change as a function of the solution conditions. This corollary is consistent with many observations with denatured proteins, and fragments, by us as well as others. Third, protein molecules such as staphylococcal nuclease or RNase A require complete folding of essentially the entire polypeptide chain before the specific stabilizing force becomes operative. Fourth, the specific force responsible for the stabilization of the three-dimensional structure of protein is of global nature and delocalized (linkage of interatomic interactions throughout the three-dimensional structure). The various aspects of the above hypothesis have been and are being tested in a number of systems.

A most useful test object is nuclease-T (an enzymatically active complex of two fragments of staphylococcal nuclease, residues 6-48 and 49-149).

Nuclease-T has been crystallized in the presence of the stabilizing ligands, pdTp and Ca^{2+}. The crystals are in the same space group as native nuclease and, from the data collected at 2-4°C to beyond 2.7 Å, are isomorphous with native crystals. Studies are in progress in collaboration with the University of Padova on Nuclease-T prepared using synthetic analogs of the 6-48 fragment. This work should yield valuable insights into degrees to which sequence may be modified without disrupting the delicate energetic balance required for stabilization. Similar research is proceeding on a complementing system of RNase A which involves three fragments. An active triple complex exhibits 28% of native activity and serves as an additional test object for the application of the principles of semisynthesis in which analogues of the fragments have been prepared. (Taniuchi, Pandin)

A. 2. Studies on cytochrome c

The application of the semisynthetic approach to the study of folding parameters for cytochrome c requires a covalent attachment of heme to the fragment that normally contains it. This attachment involves a thioether bond. It has been found that yeast cells contain an isolatable enzyme fraction which is effective in catalyzing such a reaction in the presence of NADP(H). (Taniuchi, Basile, Veloso)

A. 3. A study of the folding mechanism of cytochrome c using complementary fragments

Reactions of ferric heme-containing fragments with apofragments to form ordered complexes closely resembling native cytochrome c were studied under conditions which resolve the overall process into consecutive second order and first order kinetic steps. These reactions have been studied by the use of stopped-flow, fast kinetic techniques.

In parallel studies emphasizing equilibrium rather than kinetic aspects the stability of various complexes of cytochrome fragments has been examined using a fragment exchange technique employing radioactive forms of one or more of the associated polypeptides. This work may help establish the underlying reasons for the evolutionary invariance of many amino acid residues in cytochrome c. The second order reaction step results in species exhibiting the tryptophan fluorescence quenching of native cytochrome c and possibly containing substantial helical structure, but lacking coordination of methionine 80 to the heme iron. Methionine ligation and a substantial increase in the oxidation-reduction potential are achieved in the first order phase of the reaction. The first order kinetic steps appear to involve a substantial decrease in the conformational energy with relatively small changes in the ordering of the polypeptide chains. The complementation reactions of ferrous heme-containing fragments with apofragments were also studied. In the presence of imidazole, second order rate constants virtually identical with those for ferric heme fragments were obtained. In the absence of imidazole, the complementation reactions of ferrous heme fragments are much faster than those of the corresponding ferric heme fragments, reflecting the effect of the valence of the heme iron on the folding pathway in the absence of imidazole. (Taniuchi, Parr, Juillerat)

B. Semisynthesis of proteins including the use of enzymatic resynthesis of peptide bonds between fragments

In order to extend capabilities in the study of the effect of amino acid sequence on protein function, a novel enzymatic resynthesis method was applied to several noncovalent fragment complexes. A success in coupling non-associating peptide fragments promised the enzymatic condensation as a viable method. In order to examine the roles of local versus long range interaction in determining the conformation of S-peptide in RNase-S, X-ray crystallography method is utilized. Crystals of S-peptide fragment (11-15) suitable for high resolution work were obtained and the structure is being investigated. Further refinement of [4-F-His^{12}, 1-15]SRNase S is being carried out also.

As discussed in last year's report, substitution of water by partially nonaqueous solvents (e.g. 90% glycerol) in systems of limited proteolyses thermodynamically stabilizes the peptide bond and allows resynthesis (30-50%). A noncovalent complex of ribonuclease S-protein and 4-Fl-His^{12}-S-peptide was converted to a covalent form, allowing the study of catalysis at a low pH where the complex would have dissociated. A counterpart system where a peptide, 4-Fl-His^{119} (116-124) was chemically synthesized and complexed to ribonuclease-(1-115) provided additional information to suggest that both His^{12} and His^{119} are general catalytic acid bases. A two-fragment staphylococcal nuclease complex was "restitched" with the exclusion of loop residue, Lys^{49}. This completely abolished activity, suggesting the importance of the integrity of the loop for the correct conformation at the active site.

The crystal structure of [4-F-His^{12}, des 16-20]sRNase-S' is now being refined using the computer graphic system, GRIP of University of North Carolina. A limited x-ray diffraction data has been collected for the crystals of [4-F-His^{12}]RNase-S' analogues with a RNase substrate, UpA diffused into crystal. Structure determination work on the model S-peptide: S-protein complex has been initiated. In order to refine the structure of RNase-S [1-15]sRNase-S and [4-F-His^{12}]SRNase-S, the RNase-S structure of F.M. Richards and H.W. Wyckoff is now being refined in a collaboration with Dr. Alex Wlodawer of the National Bureau of Standards, Rockville, Md. against our Fabs. The present electron density map of [4-F-His^{12}, des16-20]-SRNase-S' generated using the calculated phases of refined RNase-S model or the phases from multiple isomorphous heavy atoms replacements data of Wychoff et al. (1970) JBC 245, 305 suggest strongly the structural identity of the active site region for native RNase-S and [4-F-His^{12}, des16-20]SRNase-S'. Through model building procedure, the above analogue structure is being refined.

As mentioned above, the inactivity of des-Lys-(6-149)-nuclease is due to the modification of the loop region integrity rather than a loss of positive charge and the same inactive des-Lys 49-(6-149) was obtained using synthetic nuclease(6-49). Active covalent semisynthetic [Gly 48] (6-149)-nuclease was obtained from enzymatically condensing synthetic [Gly 48] (6-49) with nuclease-(50-149) with trypsin. The use of 4-(oxymethyl)-Pam resin resulted in a significant increase in purity of solid phase derived peptide.

Crude peptide gave about 50% of activity when complex with nuclease-(50-149) whereas only 4% of nuclease-T activity was obtained previously using the classical chloromethyl polystyrene of solid support. (Komoriya, Homandberg, Chaiken; Collaborating individuals - D. Richardson, J. Richardson, H. Taylor, Duke University)

C. Antibodies against neurophysin and other proteins and their use for conformational and biosynthetic studies

This project is part of a general program to use specific antibodies as tools to study the chemical basis and biosynthetic origins of native protein conformation.

Purified neurophysin antibodies have been used to identify biosynthetic precursors of neurophysins (and the closely associated peptide hormones oxytocin and vasopressin) which are produced by cell-free synthesis in the presence of bovine hypothalamic mRNA. The nature of recognition of neurophysins and precursors with the purified antibodies is being studied.

Antibodies prepared against thermolysins and two large fragments of this protein have been used in immunodiffusion assays and radioimmunoassays to study conformational properties of the native protein as well as the fragments.

Affinity chromatographic techniques have been used to isolate ribonuclease antibodies specific for the S-peptide region (residues 1-20). Competition radioimmunoassay experiments are being carried out to determine specificity of the antibodies and to try to use these to design semisynthetic proteins with altered antigenicity.

In the search for a postulated neurophysin precursor, a high molecular weight cross-reactant species has been detected through immunoprecipitation from cell-free syntheses using hypothalamic mRNA. Peptide mapping has shown that the product obtained with anti-neurophysin II contains sequences analogous to those in authentic neurophysin II. Further chemical basis for the identity of the neurophysin precursor has been provided by HPLC peptide mapping. Hormone-containing sequences among the tryptic peptides of the neurophysin precursors are being sought. Brief trypsin digestion of translation mixtures directed by bovine hypothalamic mRNA appear to make the products more immunoreactive to anti-hormone antibodies. Immunoprecipitates so obtained have contained tryptic peptides which co-map with those of the authentic neurophysins. In addition, the partial purification and characterization of the mRNA coding for the neurophysins has been achieved. The existence of two separate precursors for bovine neurophysin I and II has been confirmed by the existence of two mRNA molecules differing slightly in molecular weight. The observations give chemical and immunological proof to the suggestion that the 10,000 mol. wt. neurophysins are synthesized in hypothalamic cells as parts of much larger precursors of up to 25,000 mol. wt. (Chaiken, Hough, Hargrave, Fontana)

D. Evolution of the neurophysin neurohypophyseal hormone binding site

A neurophysin-specific ligand, Met-Tyr-Phe, has been chemically synthesized and immobilized on agarose in varying concentrations and using spacers of differing lengths.

Met-Tyr-Phe-ω-aminohexyl-agarose was found to be an effective matrix for the purification of bovine neurophysins I and II.

Met-Tyr-Phe-ω-aminobutyl-agarose appeared to distinguish between previously unrecognized subpopulations of neurophysins and may thus provide an insight into possible conformational changes at the binding site(s).

Both matrices could be used to calculate binding parameters of neurophysin-ligand interactions.

As a part of a continuing study of the neurophysin-peptide hormone system, a cell-free translation system is used to obtain the radiolabelled precursors of neurophysin I and II to further investigate the processing events that surround the biosynthesis of the neurophysins. The principal techniques to be used in this study will involve passage of the translation product through two affinity ligand columns, methionyl-tyrosyl-phenylalanyl-aminohexyl agarose and lysine vasopressin agarose. The interaction of these precursors with the affinity ligands should yield information on the expression of the neurophysin hormone binding site. In the event that the translated precursors do not specifically recognize the ligands, two enzymatic preparations are known that may produce modified forms of the precursors. Microsomes from dog pancreas are reported to convert the pre-pro-neurophysin to the pro-neurophysin form. Treatment of the precursor with trypsin reportedly produces a neurophysin-like species. Differences in the interaction of these precursors with the affinity ligands may suggest a processing scheme wherein the initially translated precursor is converted to a functional neurophysin through a series of proteolytic cleavage. (Abercrombie, Chaiken)

II. Studies on normal, fetal and sickle hemoglobin

A. Hemoglobin A_o: Immunological Studies

Using non-precipitating monospecific antibodies we have studied the conformations of hemoglobin A_o in solution. Hemoglobin A_o was purified and isotopically labeled either by carbamoylation with $K^{14}CNO^{8o}$ or by reductive alkylation with tritiated borohydride. A specific subpopulation of anti-hemoglobin antibodies has been isolated by affinity chromatography using a synthetic peptide corresponding to α(129-141) and has been used in a radio-immunoassay to study conformational changes that occur at the carboxy-terminus of α chains during oxygenation. These antibodies which bind with an affinity of $5x10^8$ M^{-1} to CO-liganded ^{14}[C] hemoglobin increase the oxygen affinity of hemoglobin and, thus, act as a linked function. (J. Dean, Schechter)

B. Regulation of hemoglobin synthesis in cultures in erythroid cells

Different hemoglobins appear sequentially in the circulation during the development of many animals. These changes are brought about by differential expression of the various globin genes. The genes coding for the human non-α globin polypeptides are clustered together on a single chromosome. However the mechanism by which different genes become active at different times is not well understood. We are using the human K562 cell line as well as normal erythroid cell cultures to study this problem. To characterize and quantitate the hemoglobin synthesis occurring naturally, or after induction, in these cells under various conditions, we are using gel electrophoresis and fluorography as well as sensitive and specific radioimmunoassays for hemoglobins A and F that we have developed. Similar studies of the globin mRNA produced by these cells, and of the effect of induction of hemoglobin synthesis on the structure and arrangement of the globin genes are being carried out using cDNA hybridization. Through these studies we are describing in detail some aspects of the process of erythroid differentiation in culture. (A. Dean, Erard, Schneider, Schechter)

C. Sickle cell anemia: Deoxyhemoglobin S solubility and cell sickling studies

Intracellular gelation of deoxyhemoglobin S is believed to cause the sickling of SS erythrocytes. We have previously developed an assay using $^{13}C/^{1}H$ double-magnetic resonance techniques to quantitate the amount of polymer formed in a hemoglobin S gel. We use this technique to study intracellular gelation as a function of oxygen saturation.

We found that the amount of polymer formed within SS erythrocytes increased monotonically with decreasing oxygen saturation. Polymer could be detected at oxygen saturatoins above 90%. This was in excellent agreement with theoretical calculations based on the high intracellular hemoglobin S concentration (34 g/dl) where non-ideality effects must be considered. The curve for polymer formation as a function of oxygen saturation is less sigmoidal than published data for cell sickling; the difference is most apparent for measurements at pH 7.65. These studies indicate that cell sickling and intracellular gelation may represent different cellular phenomena. In addition, measurements of intracellular gelation should be useful in evaluating potential therapeutic agents. (Noguchi, Schechter)

D. The solubility of reconstituted hemoglobin A and S in high phosphate

We are using a solubility assay at high phosphate concentration to determine whether artificial hemoglobins A and S are different from each other. We have changed the central metal in the porphyrin of the heme group in both hemoglobins and are studying the effects of high phosphate on these reconstituted hemoglobins. These studies will allow us to determine whether any of our recent findings of subtle, but significant, conformational changes in copperk zinc, nickel and protoporphyrin IX hemoglobins A affect the solubility of this or the reconstituted hemoglobin S form. This information

is relevant to understanding the molecular mechanisms of hemoglobin aggregation, especially for sickle cell hemoglobin.

The metallohemoglobin A derivatives which contained copper(II), nickel(II), manganese(III) and reconstituted iron(III) have the same solubility as oxyhemoglobin A. Protoporphyrin IX hemoglobin A, a non-metallohemoglobin also showed no difference in its solubility from native oxyhemoglobin A. However, zinc(II) hemoglobin A showed a decrease in its solubility comparable to the solubility of deoxyhemoglobin S. The solubility of metallohemoglobin S derivatives which contained copper(II) and nickel(II) were higher than deoxyhemoglobin S but not as high as oxyhemoglobin S. Protoporphyrin IX hemoglobin S was found to have an identical solubility to that of deoxyhemoglobin S. (Alston, Schechter)

E. Sickle Cell Anemia: Design and synthesis of gelation inhibitors

Peptide inhibitors of deoxyhemoglobin S gelation have been tested using equilibrium solubility and kinetic assays of sickle hemoglobin gelation. Dipeptides which inhibit gelation do so primarily by the contribution of hydrophobic residues with L-Trp>L-Tyr>L-Phe>L-Leu>L-Ile>L-Val. Other peptides have been designed using computer graphics to study the intermolecular contacts present in the crystal structure. Tripeptides which mimic residues in the side-to-side contact have been synthesized and tested and are capable of inhibiting deoxyhemoglobin S gelation. The mechanism of action of these peptides, i.e., whether they are acting as stereospecific competitive inhibitors of sickle hemoglobin polymerization, is being studied by the X-ray difference Fourier method and spectroscopic methods to facilitate the design of better inhibitors. (Pavone, Luskey, Noguchi, Schechter)

F. Hemoglobin binding to erythrocyte membranes

It has been proposed that the interaction of hemoglobin with erythrocyte membranes has important physiological and pathological implications. An equilibrium competitive binding assay has been developed using reductive methylation to tritiate hemoglobin. We have found that in vitro binding is only detectable under non-physiological conditions, i.e., very low pH or ionic strength. It is possible that under the non-ideality conditions in the erythrocyte that some interaction occurs; however, experimental or theoretical demonstration of this remains to be accomplished. (Schneider, A. Dean, Schechter)

G. Polymerization of deoxy sickle cell hemoglobin

Polymerization of deoxy sickle cell hemoglobin molecules results in the formation of a gel phase. This gel appears to be polymorphic - in the structure of the fibers formed, and in the packing and alignment of the fibers into aggregates. To study the phase transition from solution to gel in hemoglobin and the gel structure, we have applied an osmotic stress technique. We have developed an expression for the expected behavior under osmotic stress of a solution of a non-aggregating protein and demonstrated its validity for insulin. Solution samples stressed at any osmotic pressure greater than that of

a saturated solution were converted to insulin crystals with complete removal of solvent.

Experimental methods and equilibrium tests were developed which permit osmotic stress measurements of the solution-gel transition of deoxy sickle hemoglobin. A gel phase is observed when HbS solutions are concentrated to. saturation by stressing; at greater stresses the concentration of the gel phase continued to increase gradually with stress. These results indicate a complex system in accord with other observations of polymorphism. Theoretical models for polymerization and gel structure are being tested using formulations applicable to stress data. (Prouty, Schechter)

H. Functional domains of the hemoglobin molecules and globin gene structure

The presence of non-expressed intervening sequences of DNA (introns.) in globin genes has led to suggestions that each expressed sequence codes for a domain of specific function within the protein. We plan to explore this possibility by preparing the putative domains by selective cleavage of the hemoglobin chains and characterizing each resultant peptide with reference to the known function of its amino acid residues in the intact molecule. Initial experiments with the intact chains have established improved procedures for the preparation and handling of separated globin chains, their reconstitution to hemoglobin, and their cleavage to peptide fragments. (Friedman, Schechter)

III. Studies on fertilization

A. Molecular mechanisms of sperm-egg binding

The zona pellucida contains a set of glycoproteins that are thought to mediate the species-specificity of sperm binding to oocytes. We have developed methods to isolate the zona pellucida as an intact structure and have determined its protein content. Immature mouse follicles are prepared and grown in culture for seven days. During this maturation period, the zona pellucida is formed around oocytes. The proteins of the oocyte and zona are isolated and displayed on SDS gels and two dimensional O'Farrell gels. Using a biological assay we can demonstrate that the dissolved zona will inhibit species-specific sperm binding. We are now in the process of characterizing the physico-chemical properties of zona protein and determine which of them mediate the specific sperm binding.

B. Processing of mammalian sperm nuclei by mouse oocytes

To further our understanding of the molecular mechanism of mammalian fertilization, we are also studying the structure of murine sperm chromatin and the molecular processes of nuclear decondensation that occur after sperm penetration of the egg. Using endonucleases as probes, we have documented the resistance of the condensed sperm DNA to digestion. This resistance is markedly diminished after the induction of _in vitro_ decondensation with reducing agents and proteolytic enzymes. Using our ability to decondense sperm chromatin _in_

vitro we are assaying for oocyte macromolecules that mediate the decondensation reaction. (J. Dean, Shimizu)

IV. Studies on proteins of paramecia

A. Cell-cell interactions in protozoa. Characterization of mating-type substances (M-agglutins)

Conjugation or mating in paramecia is characterized by a highly specific cell-cell interaction which appears to be mediated through receptor sites found on the cilia and surface of the pellicle (cell membrane). When cells of the opposite or complementary mating type are mixed they adhere to one another on contact forming clumps. Within minutes, the initial agglutination reaction is followed by a pairing of the complementary cells. The process of specific cell agglutination appears to be the result of a membrane associated protein or proteins called mating type substances or M-agglutinins. The project outlined in this report is designed to isolate and chemically characterize this substance(s) and define the molecular basis for the specific interaction. (Steers, Barnett)

B. Characterization of the surface antigens of "Paramecium tetraurelia"

When paramecia are placed in homologous antisera, they become immobilized and eventually die. This specific reaction has been shown to be due to a series of proteins associated with the cell surface. The antigen is distributed over 90 percent of the cell membrane and exists in a homogeneous state. While a clonal population of cells expresses only one antigen (serotype) at any given time, individual cells are capable of expressing upwards of twelve different antigens. This antigenic variation has been shown to exist now in a wide variety of species of the protozoa including both free-living forms (Paramecium) and parasitic forms (Trypanosomes). The mechanisms of antigenic variation are still unknown. An ultimate objective of this project is the partial sequence comparisons between the various antigenic types both within a single species and between several related species. (Steers)

C. Characterization of the myoglobins from Paramecium tetraurelia

The distribution of heme-containing respiratory pigments capable of reversibly binding oxygen is well documented throughout the animal kingdom. Work in our laboratory has shown that at least two protozoan species, Paramecium tetraurelia and Paramecium multimicronucleatum contain such respiratory pigments. This pigment in P. tetraurelia is present in five electrophoretically different forms which appear to be identical in molecular weight and amino acid composition and by spectral analysis. The present project is aimed at determining as much of the primary sequence of the five varient forms to allow a comparison of their primary sequence with each other and those other myoglobin species that have been sequenced to date. To date only one form, Mb-4, has been isolated in sufficient amounts and purity to allow partial sequence work to begin. (Steers, Barnett)

V. Studies on Human Interferon

A. Purification, Chemical Characterization and Chemical Synthesis of Human Interferon (Anfinsen, Bridgen, Smith, Zoon).

Two molecular weight components of human lymphoblastoid interferon have been purified and partial amino terminal amino acid sequence data obtained. Complete amino acid analysis of the two components has been performed as well as the fractionation of both cyanogen bromide and tryptic fragments of the 18,500 dalton component. The effect of exopeptidases on interferon activity has been determined. Interferon fragment 1-22 has been synthesized by Merrifield solid phase techniques. Investigation of the biological and immunological properties of this and other fragments is under way.

The purification of human lymphoblastoid interferon has been established with moderate yields of 5-8%. The procedures are capable of yields in the range of 5-15% purified material.

PURIFICATION OF HUMAN LYMPHOBLASTOID INTERFERON

Step	Interferon Units x 10^{-6}	Protein (mg)	Specific Activity (interferon units x 10^{-6}/mg protein)	Cumulative Recovery (%)
Trichloroacetic acid precipitation	800			
Sephadex G-25	690	334,756	.0021	86
Anti-lymphoblastoid interferon-Sepharose 4B	640	878	.73	80
Storage at -70°C	400			50
Ultrafiltration	220	700		28
Sephadex G-150	190	61.9	3.1	24
Ultrafiltration	140			18
SP-Sephadex[a]	110	31.9	3.4	14
Glycosidase treatment	101	32.3	3.1	13
L-tryptophyl-L-tryptophan affi-gel 10	83	7.6	11	10
Dialysis and ultrafiltration	48			6
SDS Page				
Total[b]	48			6
A	5	.065	77	0.6
B	23	.104	220	3
C	9	.101	89	1

[a] pH 5.5 eluate only.
[b] The total quantity of interferon eluted from the gels.

Sequencing of Human Lymphoblastoid Interferon: Sequence analysis of the major (18,500 dalton) component of lymphoblastoid interferon has been performed in collaboration with Drs. L.E. Hood and Michael Hunkapiller at the California Institute of Technology. Using a high sensitivity automated sequenator and an adaptation of the Edman Quadrol program (M.W. Hunkapiller and L.E. Hood, Biochemistry 17, 2124, 1978) the lower range of sensitivity approaches 10-20 pmoles in order to obtain a reproducible amino terminal sequence. Since the most material which we could successfully obtain from a typical purification was in the range of 50-80 pmoles, this sequenator system presented the only possible means of obtaining any amino terminal sequence data. The typical minimum requirement for standard sequenators or the LKB solid phase system are on the order of 10-20 times this amount. The available amino terminal sequence data is shown below:

NH_2-Ser-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Asn-Arg-Arg-Ala-Leu-Ile-Leu-Ala-Gln-Met-Gly-Arg-Ile-Ser-Leu-Phe-Ser-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Asp-Gly-Asn...

The first 25 residues in the amino terminal sequence of 3 other cyanogen bromide fragments (60-85, 112-132 and 49-165) has been determined by performing the Edman degradation reaction on a mixture of the fragments in the spinning cup sequenator and comparing the outcomes with the amino acid sequence of human leucocyte interferon (C. Weissmann, Nature, 1980, in press) which was obtained from the interferon gene DNA sequence in E. coli. Assuming a similar degree of homology between the lymphoblastoid and leucocyte interferon sequences as is seen in the first 37 residues, more than 65% of the total sequence has been obtained. Using the homology with the available leucocyte sequence there are also obvious enzymatic and chemical methods for the production of fragments of sufficient length and overlap to easily yield the entire sequence within a reasonable time.

Structural and biological differences between the 18,500 and 21,500 dalton component separated by polyacrylamide gel electrophoresis have been investigated. Initial results indicate identical amino terminal sequence residues 1-12, and fairly similar amino acid compositions.

Tryptic peptide maps of the 18,500 dalton component have been made using HPLC and will soon be compared to those of the 21,500 dalton component. Assays of relative antiviral activity of these two components on heterologous target cells (bovine kidney) or human FS-4 fibroblasts have shown no detectable difference.

In order to determine the basic structure-function relationship in the interferon molecule, the effect of exopeptidases on anti-viral activity has been determined. Aminopeptidase M has no effect on antiviral activity even after 24 hours at 37°. There appears a slight decrease in apparent molecular weight when analysed by SDS polyacrylamide gel electrophoresis. This strongly suggests that a certain portion of the aminoterminal may be removed by the enzyme and is not required for antiviral activity. Carboxypeptidase P, however, destroys 99% of the antiviral activity after 1 hour at 37°, suggesting that the carboxyl terminal portion of the molecule is essential.

B. Preparation of high specificity antisera to human interferon and then use in radioimmunoassays

Antibodies of high specificity toward human lymphoblastoid interferon have been produced in sheep and rabbits. Antibodies to interferon have also been produced by in vitro cultures of lymphocyte hybridomas. Experiments are now in progress to clone the hybrid cell lines and produce monoclonal IgG or IgM for use in radioimmunoassay and receptor studies. Using ^{3}H methylated and ^{125}I Bolton Hunter labelled interferon as labeled antigen, standard curves for quantitative immunoprecipitation assays have been made. With the present specific radioactivity of labeled interferon the radioimmunoassay can be sensitive to within 10-100 Units of interferon.

Synthetic interferon fragment 1-22 has been analyzed for its potential antigen characteristic. Although no observable anti-interferon titer was found in sheep immunized with 1-22 coupled to multichain polyalanine-lysine, labelled 1-22 is precipitated by anti-interferon and the binding can be inhibited by unlabelled crude interferon preparations. This allows the possibility for development of a quantitative radioimmunoassay using synthetic fragments as the labelled antigen.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,000-07 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Purification, Chemical Characterization and Chemical Synthesis of Human Interferon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.B. Anfinsen	Chief	LCB NIAMDD
	P.J. Bridgen	Visiting Associate	LCB NIAMDD
	D. Miller	Chemist	LCB NIAMDD
	M. Smith	Staff Fellow	LCB NIAMDD
	K. Zoon	Senior Staff Fellow	LCB NIAMDD
	D. zur Nedden	Bio-lab Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NIAMDD, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.6	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two molecular weight components of human lymphoblastoid interferon have been purified and partial amino terminal amino acid sequence data obtained. Complete amino acid analysis of the two components has been performed as well as the fractionation of both cyanogen bromide and tryptic fragments of the 18,500 dalton component. The effect of exopeptidases on interferon activity has been determined. Interferon fragment 1-22 has been synthesized by Merrifield solid phase techniques. Investigation of the biological and immunological properties of this and other fragments is under way.

Project Description:

The purification of human lymphoblastoid interferon has been established with moderate yields of 5-8%. The procedures described in the following table have been standardized and are now capable of yields in the range of 5-15% purified material.

PURIFICATION OF HUMAN LYMPHOBLASTOID INTERFERON

Step	Interferon Units x 10^{-6}	Protein (mg)	Specific Activity (interferon units x 10^{-6}/mg protein)	Cumulative Recovery (%)
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SDS Page				
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[a] pH 5.5 eluate only.
[b] The total quantity of interferon eluted from the gels.

AMINO ACID COMPOSITION OF HUMAN LYMPHOBLASTOID INTERFERON (Residues/165)

Amino Acid	Major Interferon Band	Leading Edge	Higher Molecular Weight Component
Cys	2.4	4.3	2.8
Asx	16.0	15.3	15.8
Thr	8.3	7.4	8.2
Ser	12.2	11.5	11.7
Glx	26.9	24.1	22.7
Pro	8.7	6.4	7.9
Gly	13.0	18.0	16.2
Ala	10.8	10.9	12.3
Val	9.0	9.5	10.3
Met	1.8	1.6	1.6
Ile	6.6	6.4	7.1
Leu	15.5	15.3	17.2
Tyr	4.6	4.9	4.6
Phe	7.8	8.2	8.1
Lys	11.3	10.3	10.6
His	3.9	4.6	4.8
Arg	8.8	8.9	8.8

Sequencing of Human Lymphoblastoid Interferon: Sequence analysis of the major (18,500 dalton) component of lymphoblastoid interferon has been performed in collaboration with Drs. L.E. Hood and Michael Hunkapiller at the California Institute of Technology. Using a high sensitivity automated sequenator and an adaptation of the Edman Quadrol program (M.W. Hunkapiller and L.E. Hood, Biochemistry 17, 2124, 1978) the lower range of sensitivity approaches 10-20 pmoles in order to obtain a reproducible amino terminal sequence. Since the most material which we could successfully obtain from a typical purification was in the range of 50-80 pmoles, this sequenator system presented the only possible means of obtaining any amino terminal sequence data. The typical minimum requirement for standard sequenators or the LKB solid phase system are on the order of 10-20 times this amount. The available amino terminal sequence data is shown below:

$_{3}$HN-Ser-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Asn-Arg-Arg-Ala-Leu-Ile-Leu-Ala-Gln-Met-Gly-Arg-Ile-Ser-Leu-Phe-Ser-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Asp-Gly-Asn...

The first 25 residues in the amino terminal sequence of 3 other cyanogen bromide fragments (60-85, 112-132 and 49-165) has been determined by performing the Edman degradation reaction on a mixture of the fragments in the spinning cup sequenator and comparing the outcomes with the amino acid sequence of human leucocyte interferon (C. Weissmann, Nature, 1980, in press) which was obtained from the interferon gene DNA sequence in E. coli. Assuming a similar degree of homology between the lymphoblastoid and

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Tryptic peptide maps of the 18,500 dalton component have been made using HPLC and will soon be compared to those of the 21,500 dalton component. Assays of relative antiviral activity of these two components on heterologous target cells (bovine kidney) or human FS-4 fibroblasts have shown no detectable difference.

In order to determine the basic structure-function relationship in the interferon molecule, the effect of exopeptidases on anti-viral activity has been determined. Aminopeptidase M has no effect on antiviral activity even after 24 hours at 37°. There appears a slight decrease in apparent molecular weight when analysed by SDS polyacrylamide gel electrophoresis. This strongly suggests that a certain portion of the aminoterminal may be removed by the enzyme and is not required for antiviral activity. Carboxypeptidase P, however, destroys 99% of the antiviral activity after 1 hour at 37°, suggesting that the carboxyl terminal portion of the molecule is essential.

Interferon Assays: The standard bio-assay for interferon activity is now being done in our laboratory. The assay procedure (Armstrong Applied Micro 21, 723, 1971) consists of micro titer test plates using human foreskin fibroblasts and vesicular stomatitis virus standardized against international reference standard (G-023-901-527) by semiquantitative determination of cytopathic effect. The assay can routinely be used either as a 48 hour or 24 hour assay and a new heterologic target cell line (bovine kidney cells) has also been a routine part of the interferon quantitation system.

The large scale production, purification and sequencing of lymphoblastoid interferon will continue and fragments from both the leucocyte and lymphoblastoid sequence will be synthesized and monitored for antiviral, antigenic and other biological or functional cell receptor activity.

Publications:

Zoon, K.C., Bridgen, P.J., and Smith, M.E.: Production of human lymphoblastoid interferon by Namalwa cells cultured in serum-free media. J. Gen. Virol. 44: 227-229, 1979.

Zoon, K.C., Smith, M.E., Bridgen, P.J., zur Nedden, D., and Anfinsen, C.B.: Purification and chemical characterization of human lymphoblastoid interferon. Proc. Natl. Acad. Sci. U.S.A. 76: 5601-5605, 1979.

Zoon, K.C., Bridgen, P.J., Smith, M.E., Anfinsen, C.B., and zur Nedden, D.: Purification of human lymphoblastoid interferon. Proceedings of the International Workshop on Interferon, Sloan Kettering Institute, 1980, in press.

Zoon, K.C., Smith, M.E., Bridgen, P.J., Anfinsen, C.B., Hunkapiller, M., and Hood, L.E.: Amino-terminal sequence of the major component of human lymphoblastoid interferon. Science 207: 521-526, 1980.

Zoon, K.C., and Smith, M.E.: Purification of interferon. In Kohn, L. (Ed.): Interferons. New York, Marcel Decker, in press.

Zoon, K.C., Smith, M.E., Bridgen, P.J., zur Nedden, D., and Anfinsen, C.B.: Purification and characterization of human lymphoblastoid interferon. Ann. N.Y. Acad. Sci., 1980, in press.

Zoon, K.C.: Purification and characterization of human interferon from lymphoblastoid (Namalwa) cultures. In Pestka, S. (Ed.): Methods in Enzymology. New York, Academic Press, in press.

Zoon, K.C., and Campbell, F.E.: Separation and partial characterization of Newcastle disease virus. In Pestka, S. (Ed.): Methods in Enzymology. New York, Academic Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,001-06 LCB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Characterization of the Surface Antigens of Paramecium tetraurelia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Edward Steers Jr. Research Biologist LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0		

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

When paramecia are placed in homologous antisera, they become immobilized and eventually die. This specific reaction has been shown to be due to a series of proteins associated with the cell surface. The antigen is distributed over 90 percent of the cell membrane and exists in a homogeneous state. While a clonal population of cells expresses only one antigen (serotype) at any given time, individual cells are capable of expressing upwards of twelve different antigens. This antigenic variation has been shown to exist now in a wide variety of species of the protozoa including both free-living forms (Paramecium) and parasitic forms (Trypanosomes). The mechanisms of antigenic variation are still unknown. An ultimate objective of this project is the partial sequence comparisons between the various antigenic types both within a single species and between several related species.

Work on this project is in abeyance at the present time. It will be resumed in the near future.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,002-07 LCB

PERIOD COVERED

October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Characterization of the Myoglobins from Paramecium tetraurelia.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward Steers Jr.	Research Biologist	LCB NIAMDD
	Audrey Barnett	Guest Worker	LCB NIAMDD
	Clifford E. Lee	P.S. Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The distribution of heme-containing respiratory pigments capable of reversibly binding oxygen is well documented throughout the animal kingdom. Work in our laboratory has shown that at least two protozoan species, Paramecium tetraurelia and Paramecium multimicronucleatum contain such respiratory pigments. This pigment in P. tetraurelia is present in five electrophoretically different forms which appear to be identical in molecular weight and amino acid composition and by spectral analysis. The present project is aimed at determining as much of the primary sequence of the five varient forms to allow a comparison of their primary sequence with each other and those other myoglobin species that have been sequenced to date. To date only one form, Mb-4, has been isolated in sufficient amounts and purity to allow partial sequence work to begin.

Project Description

Objectives: The objective is to extend our previous studies on the isolation and purification of the electrophoretic forms of Paramecium myoglobin and attempt sequence analysis on each species. In addition, to analyze the functional role of this protein using the methods of flash photolysis and stop-flow.

Methods Employed: Methods were adapted for large scale cultures of Paramecium on grass extracts in 5 gallon carboys. The harvested cells (usually 100 liters) were homogenized in a raffinose - phosphate buffer after which the supernatant was fractionated by ammonium sulfate precipitation. The myoglobin containing precipitate was further fractionated on Sephadex gel filtration for the heme-protein mixture. Final steps in purification are being explored using HPLC. The purification for purposes of sequence analysis utilize polyacrylamide gel electrophoresis according to the procedure of Weiner, et al. (Weiner, A.M., Platt, Terry, and Weber, Klaus, J.B.C. 247:3242-3251, 1972). Sequence analysis was carried out using a manual SDS-dansyl-Edman procedure designed for nanamole amounts of protein.

Major Findings: Preliminary results of sequence analysis on a minor myoglobin form (Mb-4) from P. tetraurelia yielded the four amino terminal residues: val-gly-ser-asp. No N-terminal data has been obtained on the other four molecular forms to date. The limited sequence for Mb-4 is homologous to that of Sperm whale myoglobin.

Significance to Biomedical Research: Many invertebrate hemoglobins and myoglobins have been identified from a variety of sources. This is the first instance of a molecule of this class appearing in the protozoa. Of special interest is the possible role of this molecule (and its variant forms) which may differ from that ascribed to these pigments in higher organisms.

Proposed Course: Continuing sequence analysis of Mb-4 and an extension of such analyses to the other four myoglobin variants found in the tetraurelia species.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,003-10 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Semisynthesis of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Komoriya	Visiting Fellow	LCB NIAMDD
	G.A. Homandberg	Guest Worker	LCB NIAMDD
	I.M. Chaiken	Research Chemist	LCB NIAMDD

COOPERATING UNITS (if any)
Department of Biochemistry, Duke Medical Center, Duke University, Durham, N.C.
Department of Chemistry, Northeastern University, Boston, MA

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.6	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to extend capabilities in the study of the effect of amino acid sequence on protein function, a novel enzymatic resynthesis method was applied to several noncovalent fragment complexes. A success in coupling non-associating peptide fragments promised the enzymatic condensation as a viable method. In order to examine the roles of local versus long range interaction in determining the conformation of S-peptide in RNase-S, X-ray crystallography method is utilized. Crystals of S-peptide fragment (11-15) suitable for high resolution work were obtained and the structure is being investigated. Further refinement of [4-F-His^{12}, 1-15]SRNase S is being carried out also.

Project Description:

Objectives: We combined two separate projects, one on enzymatic resynthesis (see G.A. Homandberg's 1978 report) and one on a study of molecular interactions using a model semisynthetic RNase-S' in order for these two aspects of protein semisynthesis to be intergrated and complement each other. We have successfully attained many important objectives on enzymatic resynthesis work in the past year.

To learn the limitations and practical considerations of this method and to develop general ideas necessary for application elsewhere, we sought a way to apply enzymatic condensation to a non-associating peptide fragment system. The success of this objective will then allow us to prepare various coupled peptide fragments free from the need for the peptides to form a stable fragment complex. Second, we sought to verify the previous finding that des-Lys 49-(6-149)-nuclease is inactive due to the modification of the loop size and that active semisynthetic (6-149)-nuclease could be made via enzymatic condensation mean using a new synthetic fragment [Gly 48](6-149). For a study on molecular interactions, active site residues containing S-peptide fragment (11 to 15) conformation was investigated. The conformation of this fragment in RNase-S complex raises a question as to what extent the interactions between S-protein and S-peptide determine and stabilize the conformation of the residues 11-15 in RNase-S. Information of this kind will help to evaluate how well one should be able to design a peptide amino acid sequence with the desired resultant conformation. The information also helps to evaluate the relative merits of "conformationally restricted peptide synthesis" for analogs of biologically active linear peptides.

Methods Employed: Conversion of protein complexes to covalent forms was investigated by SDS-gel electrophoresis. Conventional molecular sieving, ion exchange, paper and affinity chromatography methods were employed for isolation of the products. Characterization involved amino acid analysis, spectral-based substrate assays, and manual sequencing. Peptides were synthesized by the classical Merrifield solid phase technique. We also utilized a new solid support recently reported by Mitchell, A.R. et al., J. Org. Chem. 43, 2845-2852 (1978) 4-(oxymethyl)phenylacetamidomethyl-polystyrene to improve the yield and purity of the synthetic peptides.

Major Findings: 1) The inactivity of des-Lys 49-(6-149)-nuclease is due to the modification of the loop region integrity rather than a loss of positive charge and the same inactive des-Lys 49-(6-149) was obtained using synthetic nuclease(6-49). 2) Active covalent semisynthetic [Gly 48] (6-149)-nuclease was obtained from enzymatically condensing synthetic [Gly 48] (6-49) with nuclease-(50-149) with trypsin. 3) The use of 4-(oxymethyl)-Pam resin resulted in a significant increase in purity of solid phase derived peptide. Crude peptide gave about 50% of activity when complex with nuclease-(50-149) whereas only 4% of nuclease-T activity was obtained previously using the classical chloromethyl polyotyrene of solid support. 4) By the use of a kinetic trap, one can condense non-associating peptide fragments without the effect of organic cosolvents. S-peptide fragments (1-10) and (11-15) are successfully

coupled via clostripain in aqueous solution without the use of mass action to drive the condensation to completion. The success of this peptide coupling removes two significant limitations of the method, namely, the requirements for a fragment complex and a high solubility of peptides in organic cosolvents. 5) The purity of S-peptide fragment (1-10) prepared using Pam resin was also found to be higher than previously obtained where the new oxymethyl-Pam resin was not used. The fragment (11-15) was successfully crystallized suitable for X-ray analysis. 6) The crystals of S-peptide (11-15) are found to be suitable for diffraction analysis to high resolution ($\sim$1Å). The unit cell parameters are $\sim$ 16x16x16Å and the density is determined to be 1.36 g/cm^3. This crystallographic structural analysei is being carried out in a collaboration with Dr. Kay D. Onan of the Department of Chemistry, Northeastern University. 7) In a collaboration with Dr. Mark Smith of this laboratory, we began to prepare monoclonal antibody (mouse) against ribonuclease A and synthetic S-peptide (1-15). At present, we are not successful in obtaining stable colonies of clones (past 7 days after hybridization).

Significance to Biomedical Research: The success of designing a highly active amino acid sequence based on rational analysis of the native S-peptide sequence suggests strongly that it would be possible to design a peptide that would have the specified property. The present work to date strongly suggests the importance of recognizing the backbone flexibility changes as one alters native amino acid sequence. Hence, an approach of designing biologically active peptide analogues with restricted conformation achieved by appropriate amino acid substitution or cyclization should be actively investigated. Furthermore, the success of peptide fragment condensation using enzymes for the peptides that do not associate to form a complex, indicates the generality and usefulness of the enzymatic condensation method.

Appendix to Z01 AM 25,003-10 LCB
October 1, 1979 to September 30, 1980
Contracted Project

Title: Crystallographic Analyses of Active Site Residue Conformations of [4-F-His^{12}] Analogues of Ribonuclease S and Model S-peptide: S-protein Complex Conformation

Investigators: H.C. Taylor Biochemist Duke University
David Richardson Biochemist Duke University
Jane Richardson Biochemist Duke University

Department of Biochemistry, Duke Medical Center
Duke University, Durham, N.C. 27705

Summary:

The crystal structure of [4-F-His^{12}, des 16-20]sRNase-S' is now being refined using the computer graphic system, GRIP of University of North Carolina. A limited x-ray diffraction data has been collected for the crystals of [4-F-His^{12}] RNase-S' analogues with a RNase substrate, UpA diffused into crystal. Structure determination work on the model S-peptide: S-protein complex is being carried out. In order to refine the structure of RNase-S [1-15]sRNase-S and [4-F-His^{12}]sRNase-S, the RNase-S structure of F.M. Richards and H.W. Wyckoff is now being refined in a collaboration with Dr. Alex Wlodawer of the National Bureau of Standards, Rockville, M.D. against our Fabs.

Project Description:

Objectives: To determine the extent to which any induced conformational changes may be present in the active site of [4-F-His^{12}, des16-20]sRNase-S due to the fluorination of histidine 12. To obtain further refined structure of RNase-S for the structural comparison of native RNase-S with other semisynthetically derived RNase-S' complexes. With refined coordinates of RNase-S, to determine the orientation of the histidine 12 imidazole ring. The latter may be possible for [4-F-His^{12}] analogs, since extra electron density is expected for fluorine on the ring. To determine the conformation of the model S-peptide in complex for the verification of the rationales in the design of this model S-peptide.

Methods Employed: The crystals of appropriate complex are subjected to standard x-ray crystallographic analysis. The molecular graphic system, GRIP, will be utilized in model-building and refinement of structure.

Findings: The present electron density map of [4-F-His^{12}, des16-20]sRNase-S' generated using the calculated phases of refined RNase-S model or the phases from multiple isomorphous heavy atoms replacements data of Wyckoff et al. (1970) JBC 245, 305 suggest strongly the structural identity of the active site region

for native RNase-S and [4-F-His^{12}, des16-20]sRNase-S'. Through model building procedures, the above analogue structure is being refined.

Proposed Course of Action: Proceed with the refinement of [4-F-His^{12}, des16-20]sRNase-S and refining the active site residue sidechains by deleting these atoms from the initial phase calculations. Continue collecting diffraction data on [4-F-His^{12}, des16-20]sRNase-S' with UpA. Continue collecting diffraction data on the model S-peptide:S-protein complex. Determine the ring orientation of histidine 12 and compare with neutron diffraction work being carried out by Dr. Alex Wlodawer. Extend semisynthetic studies to other available analogues.

Publications:

Komoriya, A., Krigbaum, W.R. and Chaiken, I.M.: On the chemical information required in S-peptide for the conformational stabilization of ribonuclease-S. In Peptides 1978. Wroclaw, Wroclaw University Press, 1979, pp. 657-663.

Komoriya, A.: Ribonuclease as a model system for simulation. In Templeton, D. and Johnson, C. (Eds.): Proceedings of the NRCC Workshops on Stochastic Molecular Dynamics, in press.

Chaiken, I.M., Komoriya, A., and Homandberg, G.A.: Protein semisynthesis and the chemical basis of folding and function. In Gross, E. and Meienhofer, J. (Eds.): Peptide Structure and Biological Function. Rockford, Peirce Chemical Co., 1979, pp. 587-595.

Homandberg, G.A., Komoriya, A., Juillerat, M., and Chaiken, I.M.: Enzymatic conversion of selected noncovalent complexes of native or synthetic fragments to covalent forms. In Gross, E. and Meienhofer, J. (Eds.): Peptide Structure and Biological Function. Rockford, Pierce Chemical Co., 1979, pp. 597-600.

Homandberg, G.A. and Chaiken, I.M.: Trypsin-catalyzed conversion of staphylococcal nuclease-T, fragment complexes to covalent forms. J. Biol. Chem. 255: 4903-4909, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,004-07 LCB

PERIOD COVERED
March 6, 1980 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Quantitative Affinity Chromatographic Studies of Neurophysins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Angal	Visiting Fellow	LCB NIAMDD
	I.M. Chaiken	Research Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is part of a continuing program to develop the use of quantitative affinity chromatography for the characterization of macromolecule-involved interactions. The value of immobilized peptide ligands in the study of the binding of bovine neurophysins to posterior pituitary hormones is being assessed.

PHS-6040

Project Description

Objectives:
1) To develop the theory and technology of quantitative affinity chromatography to include polyvalent protein-ligand systems.
2) To resolve the question of valency in the in vivo pituitary complexes of neurophysins with the peptide hormones oxytocin and vasopressin.

Methods Employed: A neurophysin-specific ligand, Met-Tyr-Phe, has been chemically synthesized and immobilized on agarose in varying concentrations and using spacers of differing lengths.
The synthesized adsorbents have been evaluated for their usefulness in quantitative studies involving neurophysins.

Major Findings: Met-Tyr-Phe-ω-aminohexyl-agarose was found to be an effective matrix for the purification of bovine neurophysins I and II.
Met-Tyr-Phe-ω-aminobutyl-agarose appeared to distinguish between previously unrecognized subpopulations of neurophysins and may thus provide an insight into possible conformational changes at the binding site(s).
Both matrices could be used to calculate binding parameters of neurophysin-ligand interactions.

Significance to Biomedical Research: In general, quantitative affinity chromatography provides an attractive alternative to several imprecise techniques commonly used to study biologically important systems. Its increased development should allow an improved understanding of factors involved in macromolecular interactions.

Proposed Course of Action:
1) To continue to expand the use of quantitative affinity chromatography to study biologically important interactions.
2) To improve the understanding of neurophysin-hormone interactions by means of quantitative affinity chromatography.

Publication:

Chaiken, I.M.: Characterization of macromolecular interactions using quantitative affinity chromatography. In Egly, J.-M. (Ed.): Affinity Chromatography and Molecular Interaction. Paris, INSERM, 1979, pp. 373-387.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,006-07 LCB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Neurophysin-Neurohypophyseal Hormone Complexes: Biosynthetic Origin of Binding Sites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David M. Abercrombie	Guest Worker	LCB NIAMDD
	Irwin M. Chaiken	Research Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

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Laboratory of Chemical Biology

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.2	0.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

As a part of a continuing study of the neurophysin-peptide hormone system, a cell-free translation system is used to obtain the radiolabelled precursors of neurophysin I and II. The principle technique to be used involves passage of the translation product through two affinity ligand columns, methionyl-tyrosyl-phenylalanyl-aminohexyl agarose and lysine vasopressin agarose, shown by previous studies to specifically bind neurophysin I and II. The interaction of the precursors with the affinity ligands should yield information on the expression of the neurophysin hormone binding site. In the event that the translated precursors do not recognize the ligands, two enzymatic preparations are known that may produce modified forms of the precursors. Microsomes from dog pancreas are reported to convert the pre-pro-neurophysin to the pro-neurophysin form. Treatment of the precursor with trypsin reportedly produces a neurophysin-like species. Differences in the interaction of these precursors with the affinity ligands may suggest a processing scheme wherein the initially translated precursor is converted to a functional neurophysin through a series of proteolytic cleavages.

Project Description

A major objective of this project is to study the development of specific binding between the peptide hormones, vasopressin and oxytocin, and the neurophysins. Current evidence suggests that the neurophysins are biosynthesized as precursor molecules in the hypothalamus. Precursor processing is believed to occur during intra-axonal transport to the posterior pituatory where the neurophysin-hormone complexes are released. However, the stage in the processing at which the neurophysin precursor is able to specifically bind to the peptide hormones is uncertain. Information about the extent to which the pre-pro-neurophysin or the pro-neurophysin form of the precursors recognize oxytocin or vasopressin may extend the understanding of the processes that convert these precursors into functional neurophysins.

The principle technique to be used for this study involves cell-free translation of the neurophysin precursor from poly A-containing RNA isolated from bovine hypothalamus. A rabbit reticulocyte lysate translation system is used to incorporate radioactively labelled amino acids into the protein coded for by the messenger RNA added to the translation medium. The neurophysi precursors are extracted from the translation medium by immuno-precipitation with anti-neurophysin I or II antibodies purified from rabbit anti-serum. Future work for this project will involve investigating the feasibility of extracting the precursors from the translation mixture with these antibodies covalently attached to an agarose support. In addition, a new technique, known as electrophoretic desorption, will be attempted as a means of dissociating the antigen from the immunoaffinity absorbent.

Two affinity ligand columns, methionyl-tyrosyl-phenylalanyl-aminohexyl agarose and lysine vasopressin agarose, will be used to study the binding interaction between the neurophysin precursors and the hormones. Since these affinity matrices are known to specifically bind neurophysin I and II, the interaction between the precursor and the ligand will indicate the availability of the neurophysin hormone binding site on the precursor molecule. This work will be extended by studying the interaction of the precursor and the affinity ligands after cleavage of the translation product with either microsomes isolated from dog pancreas or trypsin. Dog pancreatic microsomes are reported to convert the pre-pro-neurophysin into the pro-neurophysin form, whereas trypsin reportedly cleaves the pre-pro-neurophysin into a neurophysin-like form. Differences in the binding of the various derivatives of the precursor might suggest a processing scheme whereby the initially translated precursor of neurophysin is converted to a biologically active neurophysin through a series of proteolytic steps. It will be interesting to determine whether the initial translation product has an intact hormone binding site or if enzymatic cleavage of the precursor is required to produce a structure that allows for expression of the neurophysin properties.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,007-07 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Neurophysins and Other Proteins: Conformation and Biosynthetic Precursors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.A. Hargrave	Guest Worker	LCB NIAMDD
	C.J. Hough	Guest Worker	LCB NIAMDD
	I.M. Chaiken	Research Chemist	LCB NIAMDD
	J. Seeman	Biologist	LCB NIAMDD

COOPERATING UNITS (if any)
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C. Vita, University of Padova

LAB/BRANCH
Laboratory of Chemical Biology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	2.2	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is part of a general program to use specific antibodies as tools to study the chemical basis and biosynthetic origins of native protein conformation.

Purified neurophysin antibodies have been used to identify biosynthetic precursors of neurophysins (and the closely associated peptide hormones oxytocin and vasopressin) which are produced by cell-free synthesis in the presence of bovine hypothalamic mRNA. The nature of recognition of neurophysins and precursors with the purified antibodies is being studied.

Antibodies prepared against thermolysin and two large fragments of this protein have been used in immunodiffusion assays and radioimmunoassays to study conformational properties of the native protein as well as the fragments.

Affinity chromatographic techniques have been used to isolate ribonuclease antibodies specific for the S-peptide region (residues 1-20). Competition radioimmunoassay experiments are being carried out to determine the specificity of the antibodies and to try to use these to design semisynthetic proteins with altered antigenicity.

Project Description:

Objectives:
1) To prepare antibodies specific for particular proteins.
2) To use these antibodies to study conformational properties of the native protein as well as protein derivatives and fragments.
3) To use neurophysin-specific antibodies to isolate proposed neurophysin biosynthetic precursor.

Methods Employed: Specific antibodies from rabbit antisera are detected by radioimmunoassay; purification of these is effected by neurophysin-Sepharose affinity chromatography. Purified antibodies are used in conjunction with cell-free translation studies of neurophysin biosynthesis. Wheat germ and rabbit reticulocyte lysate translations are performed with mRNA extracted from frozen bovine hypothalamic tissue, specific translation products are immunoprecipitated by antibodies, and the specifically-recognized proteins are compared with native neurophysins by peptide mapping.

For thermolysin and ribonuclease antibodies, the presence of specific antibodies is tested by immunodiffusion, radioimmunoassay, and, for the latter case, ribonuclease and S-peptide-Sepharose affinity chromatography. The radioimmunoassays also are used to test, by competition analysis, the conformational nature of particular protein fragments.

Major Findings: In the search for postulated neurophysin precursors, high molecular weight cross-reactant species have been detected through immunoprecipitation from cell-free syntheses using hypothalamic mRNA. Peptide mapping on paper has shown that products obtained with anti-neurophysins I and II contain sequences analogous to those in authentic neurophysin I and II, respectively. Further chemical basis for identity as neurophysin precursors has been provided by HPLC peptide mapping. Hormone-containing sequences among the tryptic peptides of the neurophysin precursors are being sought. Brief trypsin digestion of translation mixtures directed by bovine hypothalamic mRNA appear to make the products more immunoreactive to anti-hormone antibodies. Immunoprecipitates so obtained have contained tryptic peptides which co-map with those of the authentic neurophysins. In addition, the partial purification and characterization of the mRNA coding for the neurophysins has been achieved. The existence of two separate precursors for bovine neurophysin I and II has been confirmed by the existence of two mRNA molecules differing slightly in molecular weight. The observations give chemical and immunological proof to the suggestion that the 10,000 mol. wt. neurophysins are synthesized in hypothalamic cells as parts of much larger precursors of 23 to 25,000 and 17 to 18,000 mol. wt.

The extent to which antibody recognition requires fully native conformation is being elucidated at present in the case of anti-neurophysins by radioimmunoassay with native neurophysin and chemically or enzymatically modified neurophysin.

As indicated by immunodiffusion and radioimmunoassay experiments, a significant amount of cross-reactivity exists between thermolysin fragments

and native but not denatured protein. The immunochemical data indicate that at least one of the fragments has the propensity to form a stable conformation in solution comparable to that which the corresponding region attains in native thermolysin.

Affinity chromatographic techniques have been used successfully to isolate antibodies specific for the S-peptide region of ribonuclease. Competition experiments indicate that these antibodies can be used to distinguish between native-like and non-native states and also to distinguish native-like proteins synthetically altered to reduce the extent of antigenicity.

Significance to Biomedical Research: It is hoped that immunological investigation of neurophysin structure will lead to discovery of the biosynthetic origins and conformational nature of neurophysins and of the peptide hormones, oxytocin and vasopressin that are transported by neurophysins in mammalian systems. Studies with ribonuclease and thermolysin antibodies are aimed at the general goal of understanding the relationship between primary sequence and the attainment of native functional conformation.

Proposed Course of Action:

1) Continued investigation of conformation and biosynthesis of neurophysin and its derivatives.

2) Extension of the thermolysin studies, by preparation of purified antibodies and their use to delineate the nature of thermolysin fragments as conformationally-stable folding domains.

3) To use the purified ribonuclease antibodies specific for the S-peptide region (residues 1-20) to study conformational parameters of native and synthetically altered protein species.

Publications:

Giudice, L.C., and Chaiken, I.M.: Immunological and Chemical Identification of a Neurophysin-containing Protein Coded by Messenger RNA from Bovine Hypothalamus. Proc. Natl. Acad. Sci. U.S.A. 76: 3800, 1979.

Vita, C., Fontana, A., Seeman, J.R., and Chaiken, I.M.: Conformational and Immunochemical Analysis of the Cyanogen Bromide Fragments of Thermolysin. Biochemistry 18: 3023, 1979.

Giudice, L.C., and Chaiken, I.M.: Cell-free biosynthesis of different high molecular weight forms of bovine neurophysins I and II coded by hypothalamic mRNA. J. Biol. Chem. 254: 11767, 1979.

Hough, C.J., Hargrave, P.A., and Chaiken, I.M.: On the biosynthetic origin of neurophysin-neurohypophyseal peptide hormone complexes. In Koch, G., and Richter, D. (Eds.): Biosynthesis, Modification and Processing of Cellular and Viral Polyproteins. New York, Academic Press, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,008-17 LCB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Dynamics, Structure and Function of Protein Molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD
	G. Basile	Visiting Fellow	LCB NIAMDD
	D.C.P. Veloso	Visiting Fellow	LCB NIAMDD
	D.S. Parker	Chemist	LCB NIAMDD
	M. Pandin	Visiting Associate	LCB NIAMDD

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, Molecular Structure Section, NIAMDD
University of Padova, Padova, Italy

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Conformation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	2.7	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) As a working hypothesis a model of protein folding has been developed. (2) The three-dimensional screened precession data of crystals of Nuclease-T (an enzymically active, non-covalent complex of two fragments of staphylococcal nuclease, residues 6-48 and 49-149) bound with deoxythymidine-3',5'-diphosphate (pdTp) and Ca^{2+} have been collected at 2-4°C to beyond 2.7 A (see the report of the previous year). Phases were calculated for the observed data using both the coordinates of the intact enzyme bound with pdTp and Ca^{2+}, as reported by M.J. Legg (1977, Ph.D. dissertation, Texas A & M Univ.) and the same model partially refined against the nuclease-T data (by collaboration with G.H. Cohen, LMB, NIAMDD). The nuclease-T molecule with pdTp and Ca^{2+} can be fully traced in these maps and is highly similar to the intact nuclease model. (3) Cytochrome c synthetase catalyzing the formation of thioether bonds between heme and yeast apocytochrome c is found in a yeast mitochondrial fraction. The activity of this enzyme markedly increases by addition of NADP(H) or NAD(H) (the reduced form being more effective than the oxidized) and a heat stable postmitochondrial factor. The yeast enzyme can catalyze also the covalent bonding of heme to horse apocytochrome c.

Project Description:

Objective: To understand the mode of interatomic interactions operating in the three-dimensional structure of protein which underlies the structure, function and dynamics of protein molecules.

Methods Employed and Major Findings:

(1) A model of protein folding: the objective described above is approached in several ways in the Section of Protein Conformation as directed by the following model of protein folding which has been developing in the section (see the reports of the previous years). The model consists of four theories. First, protein molecules involve a dynamic equilibrium between the ordered (native) and the disordered (unfolded) state (this theory has been unequivocally proved using the fragment exchange technique developed in this section). Second, the disordered state is composed of conformational states which are in equilibrium with each other and therefore the number of which is assumed to be limited, that is, the disordered states are not distributed in all stereochemically possible conformations. A corollary to this theory is that the conformational distribution of the disordered state may change as a function of the solution conditions. This corollary is consistent with many observations with denatured proteins, and the fragments by us as well as others. Third, protein molecules such as staphylococcal nuclease or RNase A require complete folding of the essentially entire polypeptide chain before the specific stabilizing force would become operative, that is, a specific decrease in energy occurs in the late phase of the folding process to bind the polypeptide chain in the ordered state. This theory, deduced from the observations with a nuclease fragment, residues 1 to 126 (see the report of the previous years) is supported by the observations with the complementing fragment dependent renaturation of RNase-(1-118) containing non-native disulfide bonds (see the report of the previous years), renaturation of reduced hen egg lysozyme (see the report of the previous year from this section) and folding kinetics of the fragment complex of cytochrome c (see the report of this year by G.R. Parr and H. Taniuchi). A corollary to this theory is that the conformations of the disordered state in the ensemble would be of iso-energy and therefore the conformational transformations in this ensemble, including those involving folding to the native state, would be of statistical nature rather than to follow an obligatory pathway. This corollary is supported by the observations (reported in the previous years) that the pathway of formation of native disulfide bonds of hen egg lysozyme is multiple and varies depending on the solution conditions and that the mode of the folding kinetics of the fragment complex of cytochrome c changes as a function of the valence of the heme iron (see the report of this year by G.R. Parr and H. Taniuchi). Fourth, the specific force responsible for the stabilization of the three-dimensional structure of protein is of global nature and delocalized (linkage of interatomic interactions throughout the three-dimensional structure). This theory, which is central for the present model, has been proposed on the basis of the observations with the fragment exchange phenomenon of Nuclease-T

(an enzymically active complex composed of two fragments of staphylococcal nuclease, residues 6 to 48 and 49-149) in the presence and absence of deoxythymidine 3',5'-diphosphate (pdTp) and Ca^{2+} (see the reports of the previous years). This theory predicts that the extra stabilizing energy due to the cooperative mode of the interatomic interactions operative in the three-dimensional structure is primarily responsible for the self-ordering of te polypeptide chain, various phenomena of cooperativity characteristic of native proteins and the specificity of binding of protein with ligands or other proteins. This theory is being the focus for the experimental test in this section (see the report of this year by M. Juillerat and H. Taniuchi.

(2) X-ray diffraction studies of Nuclease-T crystals: Investigation of the dynamic properties of nuclease-T has given insight into the forces responsible for self-ordering of the polypeptide chain as described above. Although available evidence indicates that the structure of nuclease-T is similar to that of intact nuclease, a precise comparison of the three-dimensional structures of the two species is essential for the proper interpretation of the observed dynamic properties.

The structure of the intact enzyme bound with pdTp and Ca^{+2} has been described by Arnone, et al. (Arnone, A., Bier, C.J., Cotton, F.A., Day, V.W., Hazen, E.E., Jr., Richardson, D.C., Richardson, J.S. and Yonath, A. (1971) J. Biol. Chem. 246, 2302-2316) and later by Legg (Legg, M.J. (1977) Ph.D. dissertation, Texas A & M Univ.). Nuclease-T was crystallized in the presence of pdTp and Ca^{+2} in space group P41 (or P43) with cell constants a x c = 47.6 x 63.3A, a form apparently isomorphous to the nuclease crystals (see the report of the previous years). Three-dimensional screened precession data have been collected at 2-4°C to beyond 2.7 A. Phases were calculated for the observed data using both the coordinates of the intact enzyme as reported by Legg and the same model partially refined against the nuclease-T data (with the collaboration of Gerson H. Cohen, LMB, NIAMDD).

The nuclease-T molecule can be fully traced in these maps and is highly similar to the intact nuclease model. The Ca^{+2} ion and the pdTp moiety appear to be located in the expected regions.

(3) Cytochrome c synthetase: One way to understand the relationship between the amino acid sequence and the structure-function of protein would be to understand the role of the evolutionarily invariant residues. Thus, in order to investigate the role of the invariant residues of cytochrome c for the conformational dynamics and function using the fragment complementing system (see the reports of this year by G.R. Parr and H. Taniuchi and by M. Juillerat and H. Taniuchi) it is necessary to synthesize the fragments, including the heme containing fragment with substituting residues for those invariant. However, attachment of heme to apocytochrome c through the thioether bonds involves the stereochemical specificity with respect to the polypeptide chain and the α-carbons of the vinyl side chains of heme. Thus, assuming that cytochrome c is synthesized from the apoprotein and heme by cytochrome c synthetase in cells and that the enzyme permits the formation of the thioether bonds with the required stereochemical specificity, the activity of the enzyme has been investigated with the yeast cell free system (see the report of the

previous year, 201 AM 25,027 LCB).

Incubation of the ^{125}I-labeled apoprotein, prepared from ^{125}I-labeled iso-1-cytochrome c, with a yeast mitochondrial fraction in the presence of hemin, NADPH and an extract of the postmitochondrial fraction at 32 $\pm$ 1°C for 30 min has resulted in formation of a cytochrome c-like species in yields of up to 35%. This radioactive synthesized species contains a functional group which responds to reduction with ascorbate and oxidation with $k_3Fe(CN)_6$ in that it is resistant in the reduced form and susceptible in the oxidized form to trypsin action in a manner characteristic of native cytochrome c. This functional group cannot be removed from the protein by cold HCl-acetone or 8 M urea treatment. The reduced form of the synthesized species exhibits resistance against autoxidation and the oxidized form can be reduced also by cytochrome b_2. The synthesized species exhibits the same compact hydrodynamic volume of native cytochrome c. Treatment with silver sulfate followed by incubation with dithiotreitol has converted the synthesized species to the original apoprotein as judged by an increase in the hydrodynamic volume. Thus, the synthesized species is indistinguishable from the original labeled iso-1-cytochrome c by these measurements, that is, the synthesized species consists of the apoprotein to which heme is covalently attached through the thioether bond(s).

The active factor of the mitochondrial fraction is heat labile. The synthetic activity is strongly dependent on pH with a maximum approximately at pH 7.0. Hemin (or heme) appears to be required for this synthesis. The postmitochondrial fraction is inactive by itself. However, its addition markedly increases the synthetic activity. This factor is heat stable, soluble in 80% methanol (or 75% ethanol) and insoluble in ethylether or ethylacetate. Addition of NADP(H) (or NAD(H)) also increases the synthetic activity, the reduced form being more effective than the oxidized form. The postmitochondrial factor and the pyridine nucleotides appear to enhance the effect of each other. Thus, it seems that cytochrome c or a cytochrome c-like species is formed from the apoprotein and heme (or hemin) by an enzyme, cytochrome c synthetase present in mitochondria.

This yeast enzyme has been similarly found to catalyze also the formation of horse cytochrome c-like species from the horse apoprotein and heme.

(4) The three-fragment complementing system of RNase A: The biologically active, ordered three-fragment complex of protein is suitable for studies of the global force stabilizing the structure of protein as described above. Thus, the three-fragment system of cytochrome c has been developed in this section (see the report of this year by M. Juillerat and H. Taniuchi). Cytochrome c is an evolutionarily old protein. In order to compare the mode of interatomic interactions related to the stabilizing force operating in evolutionarily new protein molecules with that of cytochrome c, the three-fragment complex of RNase A is being studied. A three-fragment complex of RNase A, first prepared by Lin *et al.* (Lin, M.C., Gutte, B., Moore, S. and Merrifield, B. (1970) J. Biol. Chem. 245, 5169-5170) is composed of fragments (1-20), (21-118) and synthetic (105-124). In the present studies, fragment (21-115) is prepared from S-protein (21-124) by limited digestion with carboxypeptidase Y, followed by purification on

phosphocellulose. Fragment (116-124) has been chemically synthesized (see the report of the previous year). The three fragment complex is formed within 1 min at pH 7.1 at 25°C from S-peptide (1-20), fragment (21-115) and synthesized fragment (116-124) and exhibits approximately 28% the enzymic activity of native RNase A. The overall apparent equilibrium constant, measured on the basis of generation of enzymic activity is approximately 1.9×10^{7} M^{-2} for this three-fragment complex at pH 7.1, at 25°C.

Binding of H^{3}-labeled pdTp to nuclease fragment (1-126) in the presence of Ca^{2+} has been studied by dialysis equilibrium (see the report of the previous year). The value for the association constant is found to be 1.4×10^{2} M^{-1} at 20°C at pH 8.0 in the presence of 0.01 M $CaCl_2$.

A new batch of nuclease-T-(6-48) has been chemically synthesized (at the University of Padova) and is being purified in order to prepare semisynthetic nuclease-T containing synthetic (6-48) and native (49-149) for crystallization and possibly X-ray diffraction study.

Significance to Biomedical Research: The specificity of reactions and regulation of rate processes in cells is one of the fundamental characters of the biological system. An alteration of this control mechanism would result in malfunction of cells (a disease) unless this disorder is compensated by the system. One of the most important mechanisms of the specificity and the regulation is the specific function of globular proteins (e.g. enzymes, hemoglobins and immunoglobulins). This function of globular proteins is related to and influenced by interatomic interactions operating in the unique three-dimensional structure formed. However, the mode of operation of this interatomic interaction is not well understood. Thus, understanding the operational mode of interatomic interactions in the three-dimensional structure of proteins (e.g. the mechanism of self-ordering of the polypeptide chain in relation to the amino acid sequence) is fundamental to Biomedical Science.

Proposed Course: 1) Determination of the atomic coordinates of Nuclease-T. 2) Crystallization and X-ray diffraction studies of semisynthetic Nuclease-T' having the native sequence and analogues having substituted residues. 3) Fragment exchange studies of the three-fragment complex of RNase A in order to measure the cooperative interaction energy as a function of substituted residues using chemical synthesis. 4) Measurements of activation energy parameters of unfolding of Nuclease-T in the presence of ligands and also as a function of concentration of guanidine HCl and pH using the fragment exchange technique in order to investigate the properties of the energy barrier. 5) Development of a physico-chemical method (e.g. laser activation) for testing the hypothetical global potential function to bind the polypeptide chain in the ordered state. 6) Purification and characterization of cytochrome c synthetase.

Publications:

Acharya, A.S., and Taniuchi, H.: Influence of solution conditions on the formation of an intermediate from hen egg lysozyme containing non-native

disulfide bonds during thiol-dependent renaturation. J. Biol. Chem. 255: 1905-1911, 1980.

Acharya, A.S., and Taniuchi, H.: Preparation of a two-disulfide bonded enzymically active derivative from hen egg lysozyme. Int. J. Peptide Protein Res., in press.

Acharya, A.S., and Taniuchi, H.: On the renaturation of reduced hen egg white lysozyme containing two blocked sulfhydryl groups. Biophys. J., in press.

Basile, G., DiBello, C., and Taniuchi, H.: Formation of an iso-1-cytochrome c-like species containing covalently bonded heme group from the apoprotein by a yeast cell free system in the presence of hemin. J. Biol. Chem., in press.

The paper previously reported as in press:

Davis, A., Parr, G.R., and Taniuchi, H.: A kinetic study of the folding of nuclease B, a possible precursor of staphylococcal nuclease A. Biochim. Biophys. Acta 578: 505-510, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,011-06 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Conformational Dynamics of Cytochrome c Using a Three-Fragment Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Juillerat	Visiting Fellow	LCB NIAMDD
	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Conformation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The enzymically active order complex resembling horse heart cytochrome c can be reconstituted from heme fragment (1-25)H (residues 1 to 25) and apofragments (28-38) and (56-104) within 1 s with apparent dissociation constant $< 10^{-12}M^2$ and $< 10^{-15}M^2$ in the ferric and the ferrous form, respectively, at pH 7.0 and 25°C. Our observations have suggested a pathway in assembly of the ferric complex: (1-25)H $^{(56-104)}$ (1225)H:(56-104) $^{(28-38)}$ (1-25)H:(28-38):(56-104). Ligation of Met 80 to the heme iron seems to occur in a late phase of folding of this complex.

In order to investigate the stabilizing force of this complex using the fragment exchange technique, Met-65 and 80 of fragment (56-104) was labeled, using $[^3H]CH^3I$. While the dissociation rate of non-productive complex ferrous (1-25)H:(56-104) is very fast (half-life, less than 20 s at 0-20°C), the dissociation rate of labeled (56-104) from the three fragment complex ferrous (1-25)H:(28-38):(56-104) is slow and highly dependent on temperature (1.5×10^{-5}, 5×10^{-5}, 1×10^{-4}, 4.2×10^{-4} and 5.6×10^{-3} s^{-1} at 15°, 20°, 25°, 30° and 37°C, respectively) indicating that cooperative interatomic interactions underlie the stabilizing force.

Project Description:

Objectives: Studying the dynamics of protein molecules and the forces involved in stabilization of the tertiary structure.

Methods and Findings: Preceeding studies in this laboratory (see last annual report) showed that a three fragment complex of cytochrome c could be obtained from heme fragment (1-25)H, and apofragments (1-65) and (39-104). This fully active complex could be submited to a limited tryptic digestion and yield, after purification, an active complex composed of heme containing fragment (1-25)H, and apofragment (28-38) and (56-104). This complex can be reconstituted from the separated fragments with a 16% activity in the L-lactate dehydrogenase assay (where rate of reductions are compared to that of native cytochrome c) and can be reduced by ascorbate. An interesting comparison can be made with the nonproductive complex (1-25)H:(56-104) which cannot be reduced by ascorbate and L-lactate dehydrogenase. The visible and CD spectra are nearly identical with our three-fragment complex. Neverless a maximum at 695 nm (absent for the nonproductive complex) for the ferric three fragment complex indicate a S-Fe band characteristic to the methionine ligation to the iron atom.

Equilibrium parameter were determined at RT by titrations experiments. Affinity of heme fragment for apofragment (28-38) is low and interaction of apofragment (28-38) is weak as they are separated by gel filtration on Sephadex G50 and pH 7.0 at 8°C. On the other hand the apparent equilibrium constant of the ferric complex (1-25)H:(56-104) is 8×10^{-7} M as measured by a decrease in fluorescence intensity at pH 7.0, 25°C. Addition of a third fragment (28-38) to a mixture of fragment (1-25)H and (56-104) (molar ratio) resulted in the value for the overall dissociations constant of the three fragment complex being smaller than $10^{-12}M^2$.

Titration at 415 nm of the ferrous fragment (1-25)H (reduced by dithionite) with apofragment (56-104), shows a tightly bound complex with a dissociation constant equal to or smaller than 10^{-8} M. Further change in the visible spectra at 415 nm by adding fragment (28-38) yield a dissociation constant of 10^{-7} M or an overall constant $K(=K_1*K_2)$ being $<10^{-15}M^2$.

The ratio of each fragment in our complex was determined 1) by amino acid analysis and 2) by titrations in presence of ascorbate (where only the 3 fragment complex could be reduced) and monitoring at 415 nm. This ratio as unambiguously found to be 1:1:1. Association kinetics has been determined by stop flow experiments following absorbance changes in the soret region. An apparent second order constant of $1.7\ 10^{-6}M^{-1}s^{-1}$ was found when heme fragment (1-25)H solution was mixed with a solution of apofragments (28-38) & (56-104) or when a solution of ferric (1-25)H plus apofragment (28-38) was mixed with apofragment (56-104). A direct measure of association of fragment (28-38) to complex (1-25)H:(56-104) was possible in following absorbance changes at 695 nm. Only a first order reaction could be observed ($k = 18s^{-1}$). These results suggest that the initial association of fragment (28-38) to complex (1-25)H:(56-104) occurs in a few ms and the present experimental conditions and that ligation of Met-89 to the heme iron occurs after the initial association.

Fragment exchange technique (R.H. Hantgan, H. Taniuchi, JBC 253, 5373-5380, 1977) can be applied to the present three fragment complex. First fragment (56-104) was labeled with tritiated methyl groups on Met 65 & 80. The fragment, dissolved in ammonium acetate pH 4 containing 8M urea, was allowed to react with [^{3}H]-CH_3I. Separation from small molecular weight compound was achieved by gel filtration on column (1 x 40cm) of Sephadex G10 in 0.03M ammonium acetate pH 4. The radioactive fragment was concentrated on a SP-Sephadex C-25 column (ammonium acetate pH 4, 0.03M) and eluted with .5M Tris buffer, pH 10.2 containing 6M in Guanidine HCl and .5M in DTT. The reaction mixture (4.5ml) was standing for 22 hrs. in the same buffer and finally the fragment was dessalted on a G10 column in .3M ammonium acetate, pH 4.0. The labeled fragment was added to heme fragment 1-38H, and digesting the resulting complex (ferrous) by trypsin. The remaining complex was purified by gel filtration and the fragments were isolated by ion exchange on SP-Sephadex C-25 in 8M urea.

The labeled complexes (1-25)H:(28-38):(56-104)* and (1-25):(56-104)* were reconstituted and purified by ion exchange chromatography. Fragment exchange of ferrous complexes. Since the separation of each constituant fragment from complex (1-25)H:(56-104) and (1-25)H:(28-38):(56-104) could be achieved in quantitatively satisfactory way, an indirect method was chosen.

Based on the fact that productive ferrous complexes dissociates very slowly ($k \leq 10^{-6} s^{-1}$); a double quenching experiment was prefered. First the ^{3}H labeled complex was incubated with a large excess of heme fragment (1-38)H (which trapped all free (56-104)) and then, at different times, the reaction was quenched with a two fold excess of apoprotein (1-104). Only (56-104)* which has not been trapped by heme fragment (1-38)H is present as free fragment. A limited tryptic digestion was used to cleave the free fragments and the separation of the digested material from the complexes was achieved by gel filtration on G50 superfine at pH 7.0. Blanks (zero time as well as ∞ time) were used as a control. First order dissociations rate constants could be calculated from the above experiment (for the three fragment complex: $1.5 * 10^{-5} s^{-1}$ (15°C); $5*10^{-5} s^{-1}$ (20°C); $1*10^{-4} s^{-1}$ (25°C) $4.2\ 10^{-4} s^{-1}$ (30°C) and $5.6*10^{-3} s$ (37°C). The rate constant of unfolding of (1-25)H:(28-38):(56-104) can be related to activation parameter $\Delta H^{\ddagger}$ = 42.5 Kcal/mol. The (ferrous) two fragment complex (1-25)H:(56-104) shows a much faster unfolding since the extend of unfolding is already larger than 90% within 20 sec at 0°C (mreasured by the same technique), suggesting that stabilization of reduced cytochrome c (or complexes of cytychrome c) may be expressed by the folding rather than the unfolding rate.

Exchange of (28-38) will be the next step, clarifing the hypothetical pathway of unfolding (reversible or cyclic, non-reversible) as well as the cooperative interaction due to the addition of the third fragment. Synthetic (28-38) containing a ^{14}C labeled Phe, is synthetised by a Merrifield solid phase procedure. The peptide, after cleavage from the resin, has to be purified by gel filtration and ion exchange chromatography. Finally the synthetic peptide will combine to (1-25)H and (56-104) and purified on SP-Sephadex C-25 and used to exchange experiments.

Significance to Biomedical Research: The operational mode of interatomic interactions stabilizing the ordered structure of proteins are not well understood. Since the ordered structure of proteins is of prime importance for the biological function, understanding the mode of interatomic interactions involving the amino acid sequence folded to the three-dimensional structure if fundamental in biomedical research. This biologically active, three fragment complex of cytochrome c, now established, is expected to give an excellent test object to investigate whether a global potential function is operative in the ordered structure in order to bind the polypeptide chain. For measurements of the fragment exchange rate would give an unequivocal answer to the question as to whether the binding energy of each fragment is derived from the same global cooperative interaction. Since one of the three fragments (28-38) can be readily synthesized, the system can also be used for the study of the effect of substitution of residues on dynamics and structure-function of cytochrome c and would help in understanding the reason why the specific residues are evolutionary invariant.

Proposed Course of Research: Synthesis of labeled fragment (28-38) fragment exchange studies in order to test the binding energy between each fragment, and substitutions of several residues in fragment (28-38) by peptide synthesis in order to understand the reason why the specific residues of cytochrome c are evolutionarily in variant.

Publications:

M. Juillerat, G.R. Parr, H. Taniuchi: A biologically active, three-fragment complex of horse heart cytochrome c. J. Biol. Chem. 255: 845-853, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,013-09 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Hemoglobin Oxygen Affinity: Immunochemical Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Dean	Senior Investigator	LCB NIAMDD
	S. Harwood	Physical Science Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using non-precipitating monospecific antibodies we have studied the conformations of hemoglobin A_o in solution. Hemoglobin A_o was purified and isotopically labeled either by carbamoylation with $K^{14}CNO$ or by reductive alkylation with tritiated borohydride. A specific subpopulation of anti-hemoglobin antibodies has been isolated by affinity chromatography using a synthetic peptide corresponding to α(129-141) and has been used in a radio-immunoassay to study conformational changes that occur at the carboxy-terminus of α chains during oxygenation. These antibodies which bind with an affinity of 5×10^8 M^{-1} to CO-liganded ^{14}[C] hemoglobin increase the oxygen affinity of hemoglobin and, thus, act as a linked function.

PHS-6040

Project Description:

Objectives: This laboratory continues to maintain its strong interest in the use of monospecific antibodies as probes of protein conformation and structure. We have recently isolated an antibody population by affinity chromatography that recognizes changes in the conformation of hemoglobin A_o due to a difference in the tertiary structure between the T state (deoxy) and the R state (oxy).

Methods and Major Results: Hemoglobin A_o has been chromatographically purified on DEAE Sephadex and carbamoylated with K^{14} CNO. The extent of modification has been determined independently by hydantoin analysis and the quantitation of homocitrulline as well as by specific activity. Although structurally similar to unmodified hemoglobin, the carbamoylated protein does have a slightly higher oxygen affinity.

Sheep immunized with liganded hemoglobin A_o have produced antisera which discriminates poorly between R (oxy) and T (deoxy) forms of hemoglobin when analyzed by quantitative precipitin curves or by a double antibody radioimmunoassay. To improve the ability of the antisera to differentiate between the two states we have used affinity chromatography to isolate a subpopulation of antibodies that are specific for the carboxyl terminus of the α chain of hemoglobin. It is in this region of the chain that the penultimate tyrosine and carboxyl-terminal arginine residues become exposed to the solvent in the oxy (R) state (having been protected in the deoxy (T) conformation).

To isolate such a monospecific subpopulation of antibodies, we synthesized a tridecapaptide, α(129-141), analogous to the carboxyl terminus of the α chain by the Corley modification of Merrifield solid phase synthesis. This peptide was used to prepare a Sepharose-peptide affinity column that isolated from anti-hemoglobin antisera a protein peak that was demonstrated to contain immunoglobins by immunoelectrophoresis. Fluorescence quenching studies with these isolated antibodies showed the binding of a single antibody molecule to each hemoglobin dimer at a given time suggesting a marked restriction in the heterogeneity of the antibodies compared to the whole antiserum.

These monospecific antibodies designated α(129-141) anti-hemoglobin antibodies were then used in a direct binding assay with liganded and deoxy ^{14}C carbamoylated hemoglobin. The bound hemoglobin was separated from the free hemoglobin by a precipitating second antibody (burro-anti-sheep IgG) and the liganded state under assay conditions was confirmed spectrophotometrically. Particular care was needed in deoxygenating the dilute hemoglobin solution in order to prevent the formation of methemoglobin.

The α(129-141) anti hemoglobin antibodies showed a clear difference in their reactivity with CO and deoxy ^{14}C carbamoylated hemoglobin. They bound to the liganded hemoglobin with an affinity of $5 \times 10^8 M^{-1}$ and bound to deoxyhemoglobin with an affinity of $1 \times 10^8\ M^{-1}$ or less. We interpret the decreased binding to deoxyhemoglobin to represent the comparative

inaccessibility of the carboxyl terminus of the α chain in the T (deoxy) state. Although presumably restricted in its heterogeneity, this antibody subpopulation interacts with [^{14}C]carbamoylated hemoglobin non-linearly on a Scatchard plot which suggests that there are several monoclonal antibody populations that react with the carboxyl terminus of the α chain.

The observation that α(129-141) anti-hemoglobin antibodies react preferentially with carboxyhemoglobin suggests that they should stabilize the liganded conformation and, therefore, increase the oxygen affinity of the hemoglobin. Experimentally the presence of these antibodies increased the P_{50} of normal hemoglobin about 20% with an apparent change in the heme-heme interaction. This second phenomenon, observed in a biphasic Hill plot, can be explained either by the presence of a hemoglobin solution incompletely saturated with antibody or by heterogeneity in antibody binding affinities. The lack of any further perturbation upon the doubling of the α(129-141) anti-hemoglobin antibody concentration argues against, although it does not exclude, the first explanation. (Further increases in antibody concentration were not possible because of problems of solubility.) The second possibility is supported by the nonlinear Scatchard plot.

This type of linked reciprocal binding (i.e. antibody and oxygen) has been observed with a number of heterotropic effectors that bind preferentially to liganded hemoglobin and the thermodynamic basis of this phenomenon has been carefully detailed and quantitated by Wyman. In this construct the affinity for oxygen is changed by heterotropic effectors, such as α(129-141) anti-hemoglobin antibodies, which bind a portion of the hemoglobin that is structurally distinct from the oxygen binding site. Regardless of the mechanism of interaction such binding can be analyzed thermodynamically by the introduction of the concept of median ligand activity, P_m. This term represents the PO_2 at which the concentrations of unliganded and fully liganded hemoglobin are equal and at that point is equal to the reciprocal of the K_{assoc} of hemoglobin for oxygen. The median ligand activity takes into account any asymmetry of a Hill plot and the free energy of interactions can be calculated by $\Delta G = tRT \ln P_m$, where t is the number of oxygen binding sites.

At equilibrium in this schema, the difference in the apparent free energies of oxygen interacting with hemoglobin tetramers in the presence and absence of monospecific antibodies should be matched by the difference between the free energies of antibody binding to liganded hemoglobin vs that of binding to deoxyhemoglobin. The first two free energies can be calculated directly from the oxygen affinity curves as described above and the latter two can be ascertained from the experimentally determined binding affinities of α(129-141) anti-hemoglobin antibodies to deoxy- and liganded hemoglobin dimers. If we assume that the change of free energy of one antibody molecule binding to one antigenic determinant is the same whether the antigenic determinant is a part of hemoglobin dimer or of a hemoglobin tetramer and that the binding constants of antibody for both oxy- and carboxyhemoglobin are similar, then for two antibody molecules binding to the intact hemoglobin tetramer, $\Delta G = -2RT \ln K_{assoc}$.

Thus, if ΔG of oxygen bindings is -27.0 kcal/mole of hemoglobin tetramer in the absence of antibody and -27.9 kcal/mole of hemoglobin tetramer in the presence of antibody, then the difference between the two apparent free energies of interaction of oxygenation is 0.9 kcal/mole of hemoglobin tetramer. However, the ΔG using median association constants of antibody binding to liganded and unliganded hemoglobin is -21.7 kcal and -20.00 kcal/mole of hemoglobin tetramer respectively. Therefore, the difference in the free energies of antibody binding to liganded and deoxyhemoglobin is 1.7 kcal/mole of hemoglobin tetramer or a difference 0.8 kcal greater than we anticipated. This second quantity is a minimum difference calculated by assuming that the association constant of α(129-141) anti-hemoglobin antibodies with deoxyhemoglobin is approximately the same for all deoxyhemoglobin molecules, even though the radioimmunoassay is sensitive enough to measure the binding to only 15-18% as much deoxyhemoglobin as liganded hemoglobin. The several explanations of this apparent discrepancy in the differences in free energies are discussed in a recent publication but in any event antibody and oxygen clearly act as a linked function in binding to hemoglobin.

Significance to Biomedical Research and Proposed Course of Project:
Elegant studies by Perutz and his colleagues in the 1960's have shown significant tertiary and quarternary changes in the conformation of hemoglobin upon oxygenation. We hope that this assay using monospecific antibodies to liganded hemoglobin in solution will complement these crystallographic studies and give further information about the dynamics of this conformational shift. These antibodies could also be used to look at mutant hemoglobins with abnormal oxygen affinity in order to better define structure-function relationships. This project was terminated March 30, 1980.

Publications:

Dean, J. and Schechter, A.N.: Conformation-specific antibodies to the α chain carboxyl terminus of hemoglobin A_o. J. Biol. Chem. 254: 9185-9193, 1979.

Tack, B.F., Dean, J., Eilat, D., Lorenz, P.E., and Schechter, A.N.: Tritium labeling of proteins to high specific radioactivity by reductive methylation. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,016-07 LCB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of Hemoglobin Synthesis in Cultured Erythroid Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ann Dean	Chemist	LCB NIAMDD
	Francois Erard	Visiting Fellow	LCB NIAMDD
	Arthur B. Schneider	Guest Worker	LCB NIAMDD
	Alan N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

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SUMMARY OF WORK (200 words or less - underline keywords)

Different hemoglobins appear sequentially in the circulation during the development of many animals. These changes are brought about by differential expression of the various globin genes. The genes coding for the human non-α globin polypeptides are clustered together on a single chromosome. However, the mechanism by which different genes become active at different times is not well understood. We are using the human K562 cell line as well as normal erythroid cell cultures to study this problem. To characterize and quantitate the hemoglobin synthesis occurring naturally, or after induction, in these cells under various conditions, we are using gel electrophoresis and fluorography as well as sensitive and specific radioimmunoassays for hemoglobins A and F that we have developed. Similar studies of the globin mRNA produced by these cells, and of the effect of induction of hemoglobin synthesis on the structure and arrangement of the globin genes are being carried out using cDNA hybridization. Through these studies we are describing in detail some aspects of the process of erythroid differentiation in culture.

Project Description:

Objectives: Circulating blood contains small numbers of nucleated erythroid precursor cells which can be grown in culture to yield macroscopic clones which produce hemoglobin. Each clone represents the differentiated progeny of a single precursor cell. Our initial objective is to develop methods for the sensitive and specific assessment of total hemoglobin, and specific individual hemoglobins, HbA and HbF, produced by single clones. These will be used to examine differences between precursor cells with respect to their hemoglobin synthetic activity, as a function of cell source, time in culture and other variables.

A second line of investigation will involve establishing culture conditions for the K562 human tumor cell line. These cells grow in suspension culture and it has been previously demonstrated that they can be induced by hemin to undergo differentiation and synthesize embryonic hemoglobins and HbF. The K562 cell line offers the opportunity to study, in a homogeneous population of cells, the mechanism of induction of hemoglobin synthesis and the events accompanying erythroid differentiation. Toward this goal we will develop methods to assess quantitatively the different mRNAs and hemoglobins produced by K562 cells under a variety of conditions.

Methods Employed: Cell cultures in semi-solid media, of normal erythroid precursors have been established relying in part on previously published procedures. A method has been developed to harvest and lyse single clones of cells and measure their hemoglobin content as well as the percent composition of HbA and HbF. This procedure involves the use of specific and sensitive radioimmunoassays previously developed in this laboratory.

Culture conditions for the growth, in suspension culture, of K562 cells have been established. Hemoglobin synthesis in three subclones of the original cell line has been induced under a variety of conditions. A method for the two-dimensional analysis by isoelectric-focusing and gel electrophoresis of the hemoglobins produced by these cells has been established. The hemoglobins are identified by their pI, and relative amounts of the globin chains forming each hemoglobin are established by scanning and computer analysis of the electrophoresis pattern produced by each hemoglobin. Conditions for the propagation and use in hybridization studies of the α, β and γ cDNA probes are being established.

Major Results: Accumulation of HbF and HbA in individual erythroid clones grown in culture was measured using radioimmunoassays. Clones grown from precursors present in fetal liver, umbilical cord blood and adult peripheral blood were studied under comparable conditions to facilitate comparisons. The relative production of HbF and HbA by progenitors from different sources was appropriate for the developmental age of the source; thus, this characteristic appears to be determined in vivo. Bursts from the three sources varied in the range of percentages of HbF they synthesized. Precursors from umbilical cord blood, present during the transition from one developmental stage to another when HbF is being replaced by HbA, yielded

bursts with the greatest diversity with respect to the range of percentages of HbF they synthesized (0-100%). Clones from all sources contained between 2×10^{-14} and 1×10^{-11} moles of hemoglobin dimer. In experiments where the number of cells per burst was determined, cells contained an average of about 10 pg of total hemoglobin at the time clones were harvested.

K562 cells grow in suspension culture with a doubling time of 24 hours. Induction of hemoglobin synthesis was optimal at 30 μM hemin and production maximal at six days after induction. Only a portion of cells were induced by exposure to hemin and the induction could be successively repeated reaching the same level of hemoglobin produced for at least 3 cycles. Three subclones of K562 cells had similar induction behavior. At least six benzidine positive bands are produced by isoelectric focusing of lysates from induced cells. Three of these bands appear very faintly when lysate from uninduced cells are focused. Three of the six bonds have been identified by chain composition as hemoglobin Gower I, hemoglobin Portland and hemoglobin F. The major hemoglobins are produced in different ratios by the three subclones.

Significance to Biomedical Research: Tissue culture techniques have provided the means to study cellular regulation in a controlled laboratory environment. The study of hemoglobin synthesis in erythroid cultures should provide insight into the molecular mechanisms involved in the switch from synthesis of one hemoglobin type to another. A great deal of information is already available on the molecular structure and function of hemoglobin, as well as recently available data on the structure of the non-α globin gene locus. Consequently, this is one of the most appropriate model systems for studying eukaryotic gene regulation. Information on the cellular mechanisms operating to control synthesis of specific hemoglobins may be useful in the amelioration of thalessemia and sickle cell disease by making it possible to maintain synthesis of substantial amounts of HbF beyond the fetal period, with concomitant adjustment of other hemoglobins.

Proposed Course: K562 cells will be studied with respect to their growth characteristics and behavior after induction of hemoglobin synthesis. Identification and quantitation of hemoglobins produced by K562 subclones under various culture conditions will be continued. Induction of globin mRNA synthesis will be followed using cDNA probes and the effect of induction on globin gene structure will be studied. We will attempt to isolate and purify sufficient quantities of embryonic hemoglobins from induced K652 cultures, heretofor unavailable, to use for the production of specific antibodies for radioimmunoassays.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,021-05 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Sickle cell anemia: Deoxyhemoglobin S Solubility and Cell Sickling Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.T. Noguchi	Guest Worker	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

Laboratory of Biochemistry, NIDR (Dr. Dennis A. Torchia)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Intracellular gelation of deoxyhemoglobin S is believed to cause the sickling of SS erythrocytes. We have previously developed an assay using $^{13}C/^{1}H$ double-magnetic resonance techniques to quantitate the amount of polymer formed in a hemoglobin S gel. We use this technique to study intracellular gelation as a function of oxygen saturation.

We found that the amount of polymer formed within SS erythrocytes increased monotonically with decreasing oxygen saturation. Polymer could be detected at oxygen saturations above 90%. This was in excellent agreement with theoretical calculations based on the high intracellular hemoglobin S concentration (34 g/dl) where non-ideality effects must be considered. The curve for polymer formation as a function of oxygen saturation is less sigmoidal than published data for cell sickling; the difference is most apparent for measurements at pH 7.65. These studies indicate that cell sickling and intracellular gelation may represent different cellular phenomena. In addition, measurements of intracellular gelation should be useful in evaluating potential therapeutic agents.

Project Description:

Objectives: A quantitative description of intracellular gelation has not yet been achieved. We have previously shown that natural abundance ^{13}C NMR offers a way of studying gelation quantitatively. Using scalar-decoupling, dipolar-decoupling and proton-enhanced decoupling, the amount of free and polymerized hemoglobin can be determined. We now use this technique for studying intracellular gelation as a function of oxygen saturation.

We are also analyzing the theoretical expectation for amount of polymerized hemoglobin S under various conditions of hemoglobin concentration, temperature, oxygen saturation, and other physical variables. Most hemoglobin S solution studies use a concentration below 30 g/dl. For higher concentrations, specifically around 34 g/dl (approximate mean corpuscular hemoglobin concentration) non-ideality has a significant effect. We can use this theoretical analysis to predict the polymer fraction expected at various oxygen saturations or under other experimental conditions and compare the calculations with the actually measured quantities.

Methods Employed: Oxygenated SS erythrocytes were washed in pH 7.2 buffer and packed into 8 mm NMR tubes filled with nitrogen. (The cells were allowed to spontaneously deoxygenate while the NMR spectra were being recorded.) The oxygen saturation was monitored in a control sample using a micro blood analyzer. In order to measure polymer fraction directly as a function of oxygen saturation, SS erythrocytes were equilibrated with various gas mixtures using a tonometer (IL-237) prior to packing in the 8 mm NMR tube which was also filled with the same gas mixture.

Natural abundance ^{13}C spectra were taken using a Nicolet TT-14 spectrometer modified for high-power $^{13}C/H$ magnetic double-resonance experiments in solids. Using nitrogen gas flow through the probe in a Dewar Flask, temperature was maintained at 37^{o} with a gradient less than 2^{o} throughout the sample. A proton resonant field of 13 gauss ($\gamma_2 H_2/2\ \pi$ = 55 KH_2) was applied for dipolar decoupling. The Hartman-Hahn condition ($\gamma_1 H_1 = \gamma_2 H_2$) for proton-enhanced spectra was determined using adamantane as a standard. Relative signal intensity was determined from difference spectra.

Major Results: The polymer fraction formed due to intracellular gelation of SS erythrocytes varies roughly linearly with oxygen saturation and polymer can be detected even at high oxygen saturations, e.g. above 90%. These data are consistent with theoretical calculations based on the high intracellular concentration (~34 g/dl) and amount of liganded-hemoglobin present, which is reflected in large non-ideality effects. The curve for polymer fraction vs. oxygen saturation is less sigmoidal than that for cell sickling and the difference is most apparent at pH 7.65.

Significance to Biomedical Research: ^{13}C NMR is a useful technique for quantitating a deoxyhemoglobin S gel. The technique can also be used to study intracellular gelation under a variety of conditions such as at different pH values and at various oxygen saturations. Intracellular

gelation and cell sickling may have a different relationship to oxygen saturation and may represent different cellular phenomena. In fact, intracellular gelation may be a more meaningful parameter of the pathophysiological process in sickle cell anemia than cell morphology. Measurements of intracellular polymer should be useful in evaluating potential therapeutic agents.

Proposed Course: ^{13}C NMR will be used to study intracellular gelation of SS erythrocytes under a variety of conditions such as varying temperature and pH, and in the presence of various inhibitors of gelation. The ^{13}C NMR technique will be particularly useful in determining if inhibitors of gelation of hemoglobin S solutions are also inhibitors of intracellular gelation, a necessary requirement for any potential antigelation therapy.

^{13}C NMR will also be used to study intracellular gelation of cells from individuals with sickle trait (AS), individuals with S/β thalasemia and individuals with high persistence of fetal hemoglobin.

Refinement of these techniques will be attempted in order to study the kinetics of intracellular gelation.

Publications:

Noguchi, C.T., Torchia, D.A. and Schechter, A.N.: ^{13}C nuclear magnetic resonance quantitation of polymer in deoxyhemoglobin S gels. Proc. Natl. Acad. Sci. U.S.A. 76: 4936-4940, 1979.

Noguchi, C.T., Torchia, D.A., and Schechter, A.N.: Formation of deoxyhemoglobin S polymer in sickle erythrocytes upon deoxygenation. Proc. Natl. Acad. Sci. U.S.A., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,022-04 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Specific Interaction of Human C3 with Erythrocytes and Leukocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.F. Tack	Guest Worker	LCB NIAMDD
	P.E. Lorenz	Guest Worker	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,023-04 LCB

PERIOD COVERED
October 1, 1979- September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cell-cell Interactions in Protozoa. Characterization of Mating-type Substances (M-agglutins)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward Steers, Jr.	Research Biologist	LCB NIAMDD
	Audrey Barnett	Guest Worker	LCB NIAMDD
	Clifford E. Lee	Physical Science Technician	LCB NIAMDD

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.9	0.9

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Conjugation or mating in paramecia is characterized by a highly specific cell-cell interaction which appears to be mediated through receptor sites found on the cilia and surface of the pellicle (cell membrane). When cells of the opposite or complementary mating type are mixed they adhere to one another on contact forming clumps. Within minutes, the initial agglutination reaction is followed by a pairing of the complimentary cells. The process of specific cell agglutination appears to be the result of a membrane associated protein or proteins called mating type substances or M-agglutimins. The project outlined in this report is designed to isolate and chemically characterize this substance(s) and define the molecular basis for the specific interaction.

Project Description:

Objectives: To characterize at the molecular level the chemical basis for the specific cell-cell interaction and agglutination between complementary M-agglutinins.

Methods Employed: Culture methods have been adapted to grow large volume (100 liter) quantities of mature cells in both reactive and unreactive states as well as smaller volumes (1 liter) of immature, unreactive cells. A procedure has been developed for the isolation of reactive cilia from mature cells. A wide range of antisera has been developed against a variety of cells, subcellular fractions, and protein extracts against both unreactive and reactive cells. Immunologic analyses have been developed employing absorption tests, immunodiffusion and immunoelectrophoresis for studying the particular cellular fractions under investigation.

Procedures to isolate and purify the M-agglutinins from cellular extracts include a variety of methods including ammonium sulfate precipitation, gel filtration and ion-exchange chromatography and isoelectric focusing in sucrose gradients. The inhibitory effect of colchicine on the mating reaction has led us to attempt to isolate labeled fractions from cell extracts using this compound as a marker. Immunoaffinity chromatography has been developed as an alternate procedure for purification of the M-agglutinins. Fab fragments have been prepared from purified IgG fraction of immune antisera by methods which are now standard for this procedure.

Major Findings: Our approach to the characterization of the M-agglutinins has involved several separate procedures including the isolation of intact reactive cilia, the use of specific inhibitors of the mating reaction and the development of immune antisera which will effectively block the mating reaction.

Of several dozen agents which have been tested for their possible inhibitory effect on the mating reaction, (including various sugars, amino sugars, lectins, amino acids, peptides, and enzymes) only the proteolytic enzymes and colchicine inhibit the agglutination between complimentary cells. The results of these inhibitory studies support the conclusion that the M-agglutinins are non-conjugated proteins and that the reactive site is not associated with any other moiety.

The initial recognition by complementary cells through ciliary contact has led us to develop a separate procedure for the isolation of intact, reactive cilia free from cell bodies and cellular debris. Cilia isolated from complementary cells will agglutinate with intact cells of their respective complementary type and will even agglutinate with cilia isolated from the reactive complementary type. This procedure will hopefully allow us to identify and eventually isolate the agglutinin sites associated with the ciliary membrane.

In addition, to the above two approaches, we have developed a series of antisera against various cell types (immature, mature non-reactive and

mature-reactive) using both intact cells and cell free extracts. The IgG fraction has been purified from certain samples of the immune antisera and Fab fragments prepared and isolated for studies involving attempts to localize and map the distribution of the surface agglutinins on intact, reactive cells. Mapping of the surface agglutinins is currently underway using the Fab fragments and fluorescene-labelled antibody.

Concurrent with these studies to identify and localize the M-agglutinins, we have been carrying out a series of experiments on the purification of agglutinins from buffer extracts of reactive cells. These studies include the use of conventional purification methods as well as immuno-affinity chromatography. The results of these purification procedures has yielded fractions with M-agglutinin activity which has been purified 40 fold over the starting extracts.

In a series of experiments testing the possible "cross reactivity" of blocking antisera on other closely related species of Paramecia, we have found certain interspecies cross-reactions with respect to mating reactivity. As a result of these cross-reactions, we have developed immunoaffinity columns using antisera prepared against one species and are attempting to absorb the M-agglutinins from extracts of closely related species. This approach has the potential of removing by immunoabsorption only those antigens which exist in common between the related species.

Significance to Biomedical Research: Specific cell-cell interaction is a universal phenomenon which is important in understanding various aspects of cellular development and behavior. Mating in Paramecium is characterized by a highly specific interaction between cells of opposite mating type leading to an agglutination reaction and cell fusion. Knowledge of the molecular basis for such cell sepcificity is important in understanding a variety of cellular interactions as well as gaining an understanding of the architecture of cell surfaces associated with such reactions.

Proposed Course: The main objective of this project is the localization, purification and chemical characterization of the mating type substance(s) of Paramecia. Having identified certain cilia as the primary site of the initial recognition and agglutination reaction between complementary cells we propose to localize the sites within the ciliary membranes. In addition, localization studies are being undertaken using Fab fragments from specific immune antisera and fluorescent-labeled IgG. The inhibitory effect of colchicine on the mating reaction suggests that this compound may prove effective as a marker in isolating the M-agglutinins from cell extracts. Experiments are currently underway using tritium-labeled colchicine for this purpose.

Ultimately we hope to fully characterize the M-agglutinins by a variety of physical-chemical techniques and identify the nature of the reactive groups.

Publications:

Barnett, A. and E. Steers, Jr. Antibodies Blocking the Mating Reaction in Paramecium multimicronucleatum, syngen 2. J. Protozool. 27(1):103-107, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,025-04 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

A Study of the Folding Mechanism of Cytochrome c Using Complementary Fragments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.R. Parr	Senior Staff Fellow	LCB NIAMDD
	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD

COOPERATING UNITS (if any)

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Laboratory of Chemical Biology

SECTION
Protein Conformation Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Reactions of ferric heme-containing fragments with apofragments to form ordered complexes closely resembling native cytochrome c were studied under conditions which resolve the overall process into consecutive second order and first order kinetic steps. The second order reaction step results in species exhibiting the tryptophan fluorescence quenching of native cytochrome c and possibly containing substantial helical structure, but lacking coordination of methionine 80 to the heme iron. Methionine ligation and a substantial increase in the oxidation-reduction potential are achieved in the first order phase of the reaction. The first order kinetic steps appear to involve a substantial decrease in the conformational energy with relatively small changes in the ordering of the polypeptide chains. The complementation reactions of ferrous heme-containing fragments with apofragments were also studied. In the presence of imidazole, second order rate constants virtually identical with these for ferric heme fragments were obtained. In the absence of imidazole, the complementation reactions of ferrous heme fragments are much faster than those of the corresponding ferric heme fragments, reflecting the effect of the valence of the heme iron on the folding pathway in the absence of imidazole.

Project Description:

Objective: To elucidate the dynamic events involved in protein folding using the cytochrome c fragment complementing system.

Methods Employed and Major Findings: In previous kinetic studies of the formation of noncovalently bound, ordered complexes (closely resembling native horse heart cytochrome c) from ferric heme-containing fragments and apofragments, evidence was presented suggesting the involvement of at least two consecutive kinetic steps. It was further suggested that the initial, second order kinetic step involved interaction of tryptophan residue 59 with the heme group but that ligation of methionine 80 to the heme iron did not occur at this stage. A subsequent investigation of ordered complex formation using porphyrin containing fragments as well as methionine sulfoxide apoprotein, designed to test the role of the heme iron in the folding process, yielded results consistent with this view. We now report the separation of the second order and first order kinetic steps and the direct observation of first order kinetic processes involving ligation of methionine 80 to the heme iron. The two kinetic phases corresponding to consecutive processes in the formation of ordered ferric complexes of cytochrome c fragments have been resolved by taking advantage of the concentration dependence of the second order rate process. By using high concentrations of the fragments as well as a large excess of one fragments, the second order step has been forced to completion in very short times and thus effectively decoupled from the ensuing first order kinetic events. First order kinetic components have been observed by absorbance changes in the Soret region suggesting a change in the interatomic interactions involving the heme moiety, and at 695 nm which is specific for ligation of methionine sulfur to the heme iron. Nonproductive (biologically inactive) complexes exhibit a very similar second order reaction step but, in general, first order rate processes are not observed. Thus, nonproductive complexes are considered to mimic the intermediate complex(es) observed in the formation of productive (biologically active) complementary structures. Since productive and nonproductive ferric complexes appear to be differentiated primarily on the basis of the presence or absence, respectively, of a native-like heme environment (e.g. methionine 80 ligation), it follows that the structural details of the heme environment are established in the first order kinetic phase. The direct observation of methionine ligation as a first order kinetic process(es) is consistent with this view.

We have also investigated the kinetics of formation of productive ferric complexes in the presence of ascorbate in an attempt to determine at which stage of the reaction mechanism the standard oxidation-reduction potential is increased to a value sufficient to allow reduction by ascorbate. The results are consistent with the view that the intermediate complex(es) is not reducible by ascorbate. It follows that an increase in the oxidation-reduction potential also takes place in the first order phase of the reaction, along with ligation of methionine 80.

The dissociation rate constant for nonproductive ferric complexes at 25°C is approximately 1.0 s^{-1} while the overall dissociation rate for the productive complex (1-53)H·(54-104) as measured by a fragment exchange technique is approximately 4 x 10^{-5} s^{-1} at 25°C. Since the association rate constants differ by, at most, one order of magnitude, it follows that the first order kinetic processes observed with productive ferric complexes result in a substantial decrease in the free energy of the system with what appear to be relatively small rearrangements in the ordering of the polypeptide chains. Hence, the cooperative mode of interatomic interactions specific for the native structure appears to be established in the late phase of the folding process.

The kinetics of formation of the various complexes utilizing ferrous heme fragments as starting materials is, in the presence of imidazole, remarkably similar to the kinetics observed with ferric heme fragments in the presence of imidazole. An initial second order reaction was observed in all cases, and the existence of an additional kinetic phase was clearly indicated in the cases tested. Thus, in the presence of imidazole, the oxidation state of the heme iron has no detectable effect on the complementation reactions of heme fragments with apofragments. On the other hand, if imidazole is not present in the reaction buffer, the kinetic profiles are dramatically altered. While this is partially due to aggregation (dimerization) of the ferrous heme fragments, it can nevertheless be demonstrated that the complementation reactions with apofragments are much faster than those observed with the corresponding ferric heme fragments (in the absence of imidazole). Hence, in the absence of imidazole, changing the oxidation state of the heme iron produces substantial changes in the kinetic parameters of the complementation reactions. While we cannot make a detailed comparison of the overall reaction mechanism between the ferric and ferrous states, the increased rates of reaction of the ferrous heme fragments (as compared with the corresponding ferric heme fragments) in the absence of imidazole indicate that, at the very minimum, the transition state leading to initial complex formation must be different in the two oxidation states. If the transition states (and, therefore, the probability of formation of specific intermediates) can be altered (i.e., the energy barrier increased or decreased) by the particular experimental conditions, then it follows that the folding pathway itself is not fixed, but is susceptible to alteration, suggesting the existence of multiple folding pathways.

Significance to Biomedical Research: The understanding of the interatomic interactions underlying the conformational dynamics and functions of proteins is important in the understanding of cellular mechanisms involving proteins. These fragment complementing systems of cytochrome c are an excellent test object for analysis of the dynamic events underlying the structure and function of cytochrome c, an important class of proteins.

Proposed Course of Research:

1) An investigation of the microscopic reversibility of the folding mechanism.

2) Study the temperature dependence of the rate constants.

3) Study of the relationship of kinetic events involved in folding between native cytochrome c and the fragment complexes (the effect of pH and denaturants on the folding kinetics).

4) A study of the factors affecting the relative ratio of (1-25)H:(23-104) to that of (1-38)H:(56-104) formed on mixing (1-38)H and (1-104) (the effect of residues 39 to 65 of the heme fragment on the population ratio of the two complexes).

Publications:

Parr, G.R., and Taniuchi, H.: An investigation of ordered complex formation with chemically modified fragments of cytochrome c - The role of heme iron. J. Biol. Chem. 255: 2616-2623, 1980.

Parr, G.R., and Taniuchi, H.: A kinetic study of the formation of ordered complexes of ferric cytochrome c fragments. J. Biol. Chem. 254: 4836-4842, 1979 (Reported as in press in Z01 AM 25,025-03 LCB).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,026-03 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Solubility of Reconstituted Hemoglobins A and S in High Phosphate

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Alston	Staff Fellow	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

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Laboratory of Chemical Biology

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Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are using a solubility assay at high phosphate concentration to determine whether artificial hemoglobins A and S are different from each other. We have changed the central metal in the porphyrin of the heme group in both hemoglobins and are studying the effects of high phosphate on these reconstituted hemoglobins. These studies will allow us to determine whether any of our recent findings of subtle, but significant, conformational changes in copper, zinc, nickel and protoporphyrin IX hemoglobins A affect the solubility of this or the reconstituted hemoglobin S form. This information is relevant to understanding the molecular mechanisms of hemoglobin aggregation, especially for sickle cell hemoglobin.

Project Description:

Objectives: The gelation of hemoglobin S has been of continuing interest to this and other laboratories. We have very recently studied the immunochemical effects on a variety of metalloporphyrins reconstituted into hemoglobin A. The results showed that conformational changes had taken place in copper(II), zinc(II), nickel(II) and protoporphyrin IX hemoglobin A. We are now studying the solubility of these derivatives in hemoglobin A and S in a high phosphate assay to see whether the conformational changes have affected their solubility. The high phosphate assay is well established for testing the tendency of different hemoglobins especially sickle hemoglobin, to aggregate.

Methods Employed: Hemoglobins A and S were purified from the blood of AA, SS, and SC donors by ion exchange chromatography. Normal hemoglobins A and S were reconstituted using copper, cobalt, zinc, manganese, nickel, protoporphyrin IX and the native iron. This was accomplished by removing the heme group yielding the apohemoglobin and reconstituting the protein with the above metalloporphyrin derivatives. The various metallohemoglobins were purified by column chromatography employing Sephadex and carboxymethyl cellulose. The hemoglobins were then concentrated, dialyzed against 0.05 M phosphate buffer, pH 7.1, frozen in liquid nitrogen and stored in a Revco freezer.

Major Results: The metallohemoglobin A derivatives which contained copper(II), nickel(II), manganese(III) and reconstituted iron(III) have the same solubility as oxyhemoglobin A. Protoporphyrin IX hemoglobin A, a non-metallohemoglobin also showed no difference in its solubility from native oxyhemoglobin A. However, zinc(II) hemoglobin A showed a decrease in its solubility comparable to the solubility of deoxyhemoglobin S. The solubility of metallohemoglobin S derivatives which contained copper(II) and nickel(II) were higher than deoxyhemoglobin S but not as high as oxyhemoglobin S. Protoporphyrin IX hemoglobin S was found to have an identical solubility to that of deoxyhemoglobin S.

Significance to Biomedical Research: The various metallohemoglobin derivatives serve as unique probes in studying the active site of the native hemoglobin molecule and the concomitant effects transmitted to the rest of the protein moiety. These studies will help us understand what occurs when the iron atom in the heme group of native hemoglobin changes its electronic properties when undergoing the R T transition. The various metalloporphyrin derivatives allows us to study the effects of changes in the ionic radius, electron spin, coordination sphere and spin density at the active site on the remainder of the protein molecule.

We showed previously by immunochemical techniques that subtle but significant changes had taken place in some of the reconstituted derivatives. These changes were manifest in some of the antigenic determinants on the

surface of the hemoglobin molecule. The high phosphate assay will allow us to ascertain whether any of the intermolecular contact points which exists in the deoxyhemoglobin S gels are affected by these conformational changes. These metal substitutions are thus an excellent way in which to modify subtly the surface of the hemoglobin molecule in order to identify regions of functional importance in the polymerization process.

Proposed Course of Project: We are continuing to test the solubility of these reconstituted hemoglobin derivatives, of hemoglobin A and S, in high ionic strength phosphate buffers and are preparing to grow crystals of each form so that we may be able to locate the sites which have undergone conformational changes. If we can identify sites in which such conformational changes significantly inhibit gelation, we will synthesize compounds (especially peptides) which are complementary to these sites to see if they have a high degree of specificity in inhibiting gelation.

Publications:

Alston, K., Dean, A., and Schechter, A.N.: The Immunochemical Effects of Metal Substitution in the Prosthetic Group of Hemoglobin. Molecular Immunology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,027-03 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Cytochrome c Biosynthesis: Enzymatic Formation of Heme-apoprotein Covalent Bond

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Basile	Visiting Fellow	LCB NIAMDD
	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Conformation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This report has been combined with Z01 AM 25,008 LCB.

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,028-02 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Sickle Cell Anemia: Design and Synthesis of Gelation Inhibitors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. Pavone	Visiting Fellow	LCB NIAMDD
	K.L. Luskey	Research Associate	LCB NIAMDD
	C.T. Noguchi	Guest Worker	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)
Division of Computer Research and Technology, NIH (Dr. R.J. Feldmann)
Medical Research Council Laboratory of Molecular Biology, Cambridge, England (Drs. S.E.V. Phillips and M.F. Perutz)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Peptide inhibitors of deoxyhemoglobin S gelation have been tested using equilibrium solubility and kinetic assays of sickle hemoglobin gelation. Dipeptides which inhibit gelation do so primarily by the contribution of hydrophobic residues with L-Trp>L-Tyr>L-Phe>L-Leu>L-Ile>L-Val. Other peptides have been designed using computer graphics to study the intermolecular contacts present in the crystal structure. Tripeptides which mimic residues in the side-to-side contact have been synthesized and tested and are capable of inhibiting deoxyhemoglobin S gelation. The mechanism of action of these peptides, i.e., whether they are acting as stereospecific competitive inhibitors of sickle hemoglobin polymerization, is being studied by the X-ray difference Fourier method and spectroscopic methods to facilitate the design of better inhibitors.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Sickle hemoglobin under physiological conditions aggregates upon deoxygenation to form a viscous gel composed of long fibers. This form of aggregation within an SS erythrocyte is believed to result in the cell deformity and ridigity characteristic of a sickle SS erythrocyte. Much effort has been devoted in the last years to identify the residues involved in the protein-protein interactions which might be responsible for the stability of the gels.

Since crystallization and formation of gels are both processes of aggregation, a comparison has been attempted between the three dimensional structure of crystals of deoxyhemoglobin S and the results of electron microscopy and x-ray diffraction of fibers. Presently it seems reasonable to believe that the interaction sites among tetramers found in the crystals are quite similar to that in the gels. In searching for a therapeutic agent for sickle cell anemia we are looking for an additive which will specifically bind to the hemoglobin surface at the sites of interactions as found in the crystals and which could change the kinetic and/or the equilibrium between the free protein molecules and the gel.

Using high resolution structural information available from the crystal structure of deoxyhemoglobin S, we should be able to define regions in the intermolecular contact areas between two neighboring hemoglobin molecules to which we might design a peptide which would bind to this region and compete with the hemoglobin self-association.

Furthermore, we have previously shown that the aromatic amino acids, phenylalanine and tryptophan, as well as peptides containing phenylalanine inhibit gelation. To better characterize the effects of these compounds, we are studying dipeptides with residues of varying hydrophobicity in the carboxyl-terminal position and residues with charged side chains in the amino-terminal position. These compounds are much more soluble than the aromatic amino acids and allow us to study their effects over a broad range of concentration. We have also noted that the behavior of peptides which decrease the solubility of deoxyhemoglobin S is analogous to the non-ideal behavior of concentrated deoxyhemoglobin S solutions when large molecules such as CO-hemoglobin or bovine serum albumin are present. To characterize the non-ideal effect of peptides we have studied oligomers of amino acids which do not inhibit gelation.

These studies of the effects of hydrophobic residues and of non-ideality of peptides on deoxyhemoglobin S gelation should provide useful information in the rational design of a potent stereospecific inhibitor of sickle hemoglobin gelation.

Methods and Major Results: We have used the Display Graphics System of the Division of Computer Research and Technology to study the molecular structure of the deoxyhemoglobin S crystal. The coordinates were supplied by Dr. W. Love of Johns Hopkins University based on a 3 Å map of the protein. The surfaces of the hemoglobin S molecules at regions of intermolecular contacts in the crystal were examined and two of these regions, around β^{73}

and β^{22}, were selected as being the most suitable for building complementary peptides.

The region surrounding glutamic acid β^{22} is involved in the axial (up-down) contact of hemoglobin molecules within the double stranded structure of the crystal. Six peptides of 3-8 residues which were designed to interact with this region were synthesized and tested. Unfortunately, none of these peptides inhibited gelation and they all decreased deoxyhemoglobin S solubility, similar to the non-ideal effect of large peptides.

The other major area of intermolecular contact is the side-to-side contact. In this region the β^{6} valine fits into a hydrophobic pocket on the neighboring hemoglobin molecule where phenylalanine β^{85} and leucine β^{88} are located. The β^{4} threonine forms a hydrogen bond with the β^{73} aspartic acid on the neighboring molecule. The peptide β^{s} 4-6 (L-Thr-L-Pro-L-Val) was synthesized by Merrifield solid-phase techniques but did not inhibit gelation while the analogous tripeptide L-Thr-L-Pro-L-Phe, synthesized with the same procedure, in which the carboxy-terminal valine residue is substituted with the more hydrophobic phenylalanine residue, had a significant inhibitory effect.

In order to determine the reasons for such differences of these two very similar compounds, we have synthesized and tested model tripeptides with different structural features: i) blocked end charges, ii) backbone flexibility and iii) hydrophobicity of the side chains. Thus we have synthesized by solution methods two groups of peptides, namely: (IBA = Isobutylamide, PEA = Phenethylamide) A) β-Ala-L-Pro-IBA, β-Ala-Gly-IBA, Gly-L-Pro-IBA, Gly-Gly-IBA and B) β-Ala-L-Pro-PEA, β-Ala-Gly-PEA, Gly-L-Pro-PEA, Gly-Gly-PEA. Both DCC and/or succinimide active esters were used in the coupling step of the syntheses; intermediate compounds were purified by two phase extraction and crystallization and the final products were purified by ion-exchange and gel filtration chromatography. Satisfactory yields were obtained for most of the synthesized compounds and final purity was ascertained by amino acid analysis and thin layer chromatography.

We have interestingly found that the carboxy-terminal charge has no significant effect on the inhibitory capacity of the tripeptides containing phenyl groups, while tripeptides containing isobutyl groups as hydrophobic part of the molecule have inhibitory effects only when the carboxy-terminal charge is absent. No particular backbone conformation seems to be required for these tripeptides in order to increase deoxyhemoglobin S solubility since the substitution of the middle amino acid proline with a glycine does not change their effectiveness. Furthermore, we have found that whether a threonine, glycine or β-alanine residue is present in the amino-terminal position no significant changes in the inhibitory capacity of these tripeptises is observed, while the compounds containing the isobutyl group at the carboxy-terminal position are less effective than those containing phenyl groups. We have also studied amino acids over a large concentration range. The aromatic amino acids inhibit gelation (approximately 6% increase in solubility at 10 mM and the aliphatic amino acids leucine and isoleucine have probably a very small inhibitory effect. L-arginine also increases deoxyhemoglobin S solubility (approximately 10% at 100 mM) while L-Lys,

L-Asp, Gly and L-Val had a negative effect. We have attempted by differential absorption and fluorescence spectroscopy to determine the mechanism of action of these compounds but so far no change of the spectral properties of both hemoglobin and additive have been detected. A possible reason for this might be the weak binding of these amino acids to the hemoglobin and their limited solubility.

For these reasons we have tested by the sedimentation equilibrium assay dipeptides containing at the amino terminal position L-Arg, L-Lys, Gly or L-Asp and at the carboxyterminal position L-Trp, L-Tyr, L-Phe, L-Ile, L-Leu or L-Val. The charged residue served mainly to solubilize the compound although, in some cases, L-Arg complemented the inhibition shown by the rest of the molecule. The hydrophobic residue was the primary determinant of effectiveness of the dipeptides with the relative effectiveness of the carboxy-terminal being L-Trp>L-Tyr>L-Phe>L-Leu>L-Ile>L-Val. This order is mainly related to the hydrophobicity of these residues although this is not the sole determinant. Dipeptides with L-Tyr were more effective than those with L-Phe, although L-Phe is more hydrophobic. Further modifications of these dipeptides were studied by synthesis of L-Lys-phenethylamide and L-Lys-isobutylamide. These compounds differ from L-Lys-L-Phe and L-Lys-L-Val by the absence of the carboxy-terminal group. L-Lys-phenethylamide was only slightly more effective than L-Lys-L-Phe; however, L-Lys-L-Val had no inhibitory effect on deoxyhemoglobin S gelation whereas L-Lys-isobutylamide increased solubility 18% at 100 mM.

At present the exact mechanism of the inhibition of gelation is uncertain. We are now working with Drs. S.E.V. Phillips and M.F. Perutz of the MRC Laboratory of Molecular Biology to determine the binding sites of these peptides by difference Fourier analysis from X-ray crystallography.

In order to better understand the behavior of peptides in a concentrated solution of hemoglobin S, a series of experiments was carried out in which increasing amounts of amino acids and short homopolymers of amino acids were added to hemoglobin S and the kinetics of gelation and equilibrium solubility were measured. Lysine, di-lysine, tetra-lysine, $lysine_{8-12}$, serine and hexa-proline all behaved remarkably similar when the effect on hemoglobin S solubility is compared with relation to the weight amount of additive. This behavior is very similar to the effect observed by others of large globular proteins increasing the activity coefficient of hemoglobin S and thus decreasing its solubility. This behavior of these model compounds provide a background against which peptide additives must be compared to determine their effect on gelation. Thus, proline when compared to these other amino acids does not exhibit a great decrease in solubility of hemoglobin S and thus may interact with the protein and inhibit gelation, although to a minor degree.

Significance to Biomedical Research: The intracellular gelation of sickle hemoglobin is the basis of the severe disease, sickle cell anemia. Recent progress in understanding the molecular basis of gelation and the structure of the deoxyhemoglobin S gel and crystal has opened the possibility of designing specific inhibitors of gelation which might have ultimate therapeutic importance. We are studying peptides complementary to the

surface of the hemoglobin molecule for this purpose. The problem is a difficult one because of the very high hemoglobin concentration in the cell and the resulting non-ideality but it may be possible to find classes of peptides with significant inhibitory effect, greater than that of free phenylalanine for example. However, possible problems with cell permeability, toxicity, and biological life-time will also have to be considered. At present, this approach is among the most promising in this disease and serves as a general model for the inhibition of protein-protein interactions in other systems.

Proposed Course of Research: We plan to continue our strategy of molecular modeling to design and synthesize peptide inhibitors of deoxyhemoglobin S gelation. In addition, it is necessary to clearly define the mechanism of action of these compounds. We will use the X-ray difference Fourier method and nuclear magnetic resonance to attempt to identify binding of these compounds to hemoglobin.

Publications:

Schechter, A.N.: Stereospecific Inhibitors of the Gelation of Sickle Hemoglobin. In Huisman, T.H.J. and Miwa, S. (Eds.): Symposium on Abnormal Hemoglobins and Red Cell Enzymes, Hemoglobin, in press.

Luskey, K.L., Pavone, V., Noguchi, C.T. and Schechter, A.N.: Peptides as Inhibitors of the Gelation of Sickle Hemoglobin. In Sigler, P.B. (Ed.): Molecular Basis of Mutant Hemoglobin Dysfunction. New York, Elsevier-North Holland, in press.

Thomas, K.A. and Schechter, A.N.: Protein Folding: Structural, Evolutionary and Chemical Aspects. In Goldberger, R.F. (Ed.): Biological Regulation and Development, Vol. 2, New York, Plenum Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,029-02 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Molecular Mechanism of Sperm-Egg Binding

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Dean	Senior Investigator	LCB NIAMDD
	S. Shimizu	Visiting Fellow	LCB NIAMDD
	S. Harwood	Physical Science Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.3	0.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The zona pellucida contains a set of glycoproteins that are thought to mediate the species-specificity of sperm binding to oocytes. We have developed methods to isolate the zona pellucida as an intact structure and have determined its protein content. Immature mouse follicles are prepared and grown in culture for seven days. During this maturation period, the zona pelluuda is formed around oocytes. The proteins of the oocyte and zona are isolated and displayed on SDS gels and two dimensional O'Farrell gels. Using a biological assay we can demonstrate that the dissolved zona will inhibit species-specific sperm binding. We are now in the process of characterizing the physico-chemical properties of zona protein and determine which of them mediate the specific sperm binding.

Project Description:

Objectives: We are interested in examining the molecular processes of mammalian fertilization and early development. As a first step in this longer term project we are undertaking to isolate and characterize the proteins in the murine zona pellucida that presumably mediate the species-specific sperm-egg interaction.

Methods and Major Results: At birth ova in the female mouse are in mieotic arrest and have no potential for mitotic cell division prior to fertilization. This leads to a paucity of biological material which can be partially overcome by radioactively labeling the protein in the maturing oocyte. To obtain zona proteins we have modified previously published methods for the short term in vitro culture of mouse follicles. Two to three thousand follicles are isolated from 8 day old DBA/2 females and grown in culture for seven days in such a manner that the granulosa cells spread in a monolayer and trap the oocytes underneath. During this period the eggs enlarge to a diameter of 65-70 μm and undergo a maturation process which includes the production of the zona pellucida. The zona is an acellular matrix, thought to be composed of glycoproteins, which surrounds the oocyte and has a diameter of approximately 5-7 μm.

We have developed methods for the isolation of the zona pellucida as an intact structure and have used three criteria for its purity. First we have examined the end product of the various procedures described below by high resolution Nomarski optics microscopy. Second, we have tested for the absence of the cytoplasmic enzyme lactic dehydrogenase and the plasma membrane ATPase and third we have examined the protein composition by one dimensional SDS gels and two dimensional O'Farrell gels.

Because of the small amounts of material available to us, this latter criteria requires the radioactive labeling of the proteins. To avoid possible artifacts induced by post-synthetic labeling procedures, we have chosen to label the egg and zona proteins in culture with radioactively amino acids (^{35}S-methionine, ^{35}S-cysteine) or carbohydrates (^{3}H-glucosamine, ^{3}H fucose). The proteins can then be analyzed by one dimensional SDS electrophoresis or by two-dimensional O'Farrell gels using isoelectric focusing in the first dimension and SDS slab gels in the second dimension. Fluorography, according to the methods of Bonner and Laskey is used to detect radioactive proteins.

Using a published procedure we attempted to isolate the intact zona by gentle douncing followed by repeated washes in culture media. Mophologically the zona appeared pure and there was less than 1% of intact eggs LDH activity. However, more than 30% of the intact eggs ATPase activity remained and multiple radioactive protein bands were visible after electrophoresis and fluorography. By washing the eggs in 0.2% NP-40 after douncing, the ATPase activity disappeared in the zona preparation although it remained at its control level in the lysed egg in the presence or absence of 0.2% NP-40. Moreover, only three protein bands labeled with ^{35}S-methionine appeared

after electrophoresis with sharp bands appearing at 185,000, 47,000 daltons and a diffuse band at 135,000 daltons. All three proteins appeared to have acid pI's based on their mobility in the isoelectric focusing dimension of O'Farrell gels. Comparable results were observed in morphologically clean and intact zona pellucidae obtained by freeze-thawing oocytes in the presence of 0.2% NP-40.

Using o-phthalaldehyde we have measured 12ng of protein in the intact egg and it appears that more than 20% of this is in the zona pellucida. However, the amount of radioactive amino acid and sugar incorporated into the zona proteins as a percent of the total egg content varies widely from 5% for ^{35}S-methionine to 75% for ^{3}H-fucose. We are now investigating whether the use of a variety of different labeled amino acids and sugars will reveal the same or different protein patterns and whether these patterns change over the course of the culture period.

We have established a biological assay to measure sperm binding to the intact mouse egg. By inducing super-ovulation with gonadotrophins, 20-40 mature eggs can be obtained from each mouse and used to ascertain the ability of capacitated sperm to bind to the zona pellucida surrounding the egg. Having determined that the isolated intact zona will bind sperm, we have demonstrated that this structure can be dissolved either by heating to 65^{o} for 60 minutes or by acidic pH and that the dissolved material will inhibit sperm binding to the intact egg. We are in the process of ascertaining which of the three proteins that we have identified in the zona is responsible for this inhibitory action for the dissolved zona. Presumably one of them acts as the sperm binding protein in the intact zona pellucida and conveys the species-specificity of this interaction.

Significance to Biomedical Research and Proposed Course of Project:

An understanding of the molecular basis of mammalian fertilization would open the possibility of modulating the sperm-egg interaction. This in turn could have profound practical application in animal husbandry and, perhaps, in development of more effective means of prevention of world overpopulation.

In a biochemical sense this project represents a system in which cell-cell interaction and the proteins that mediate these specific interactions can be well studied. The oocyte proteins may act as receptors and bind sperm surface ligands in a reversible manner or they may irreversibly modify sperm surface proteins so as to facilitate binding and penetration of the zona pellucida by the sperm. Subsequent fusion of the sperm with the oocyte represents an initial point in embryogenesis and cell division. Thus, murine fertilization is a model system of cell differentiation and early mammalian development. Studies on changes in surface and cytoplasmic proteins can be undertaken so as to begin to sort out the complex variables that regulate early mammalian differentiation.

We hope to use the in vitro culture of mouse oocytes not only to isolate and identify the function of the proteins in the zona pellucida but also to investigate the site and regulation of their synthesis. We also hope to be able to use the isolated zona proteins as probes to investigate

sperm acrosomal proteins that are involved in fertilization. More long range projects will include examination of sperm penetration of the zona pellucida, sperm-egg plasma membrane fusion and the subsequent cortical reaction that presumably modifies zona proteins and prevents polyspermy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,030 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Purification and in vitro Translation of Human Lymphoblastoid Messenger RNA.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: K. Zoon Staff Fellow LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been combined with Z01 CB 08212-06 LPP.

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,031-02 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Preparation of High Specificity Antisera to Human Interferon and Then Use in Radioimmunoassays

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Smith	Staff Fellow	LCB NIAMDD
	D. zur Nedden	Bio-lab Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies of high specificity toward human lymphoblastoid interferon have been produced in sheep and rabbits. Antibodies to interferon have also been produced by in vitro cultures of lymphocyte hybridomas. Experiments are now in progress to clone the hybrid cell lines and produce monoclonal IgG or IgM for use in radioimmunoassay and receptor studies. Using ^{3}H methylated and ^{125}I Bolton Hunter labelled interferon as labeled antigen, standard curves for quantitative immunoprecipitation assays have been made. With the present specific radioactivity of labeled interferon the radioimmunoassay can be sensitive to within 10-100 Units of interferon.

PHS-6040

Project Description:

Objectives: Antibodies to human lymphoblastoid interferon have been produced in sheep for the past three years. Recently, only highly purified interferon preparations have been used as immunogen in the sensitized sheep with resultant production of significantly higher titer of antibody and much greater specificity than was previously obtained. Sheep are subjected to plasmaphoresis and the plasma is clotted and delipidized with dextran sulphate. Dilutions of $1/10^6$ are capable of 50% neutralization of interferon activity. IgG has been purified from this antiserum and used to make an immunoadsorbent affinity columns. This new column had a 10-fold increased capacity of interferon binding per ml of sepharose and an apparent association constant 3 to 4 magnitudes greater than those previously used in the purification of interferon. Some difficulty has been observed in recovering all of the interferon activity applied to the column, however, several different elution conditions are presently being evaluated to increase recovery and to take advantage of the increased affinity and specificity.

Procedures aimed at attaining high specific radioactivity interferon which still retains most or all of its antigenic character have resulted in two successful methods. Using reductive methylation with NaB^3H_4 (specific activity 500 curies/mmole) high specific activity interferon (10^8 dpm/µg) has been made which retain antigenic activity but lose all antiviral activity. Using ^{125}I-Bolton Hunter reagent similar specific radioactivity can be obtained but with retention of both antigenic and antiviral properties. Using purified sheep IgG anti-interferon and both types of labelled interferon, immunoprecipitation assays and other preliminary work for the generation of a quantitative radioimmunoassay for interferon has been done. With this specific radioactivity, the interferon radioimmunoassay will be sensitive to within 1 to 2 orders of magnitude of the present bioassay for antiviral activity. The primary problem with the bioassay is the high standard error and this will be dramatically reduced with the use of a radioimmune assay.

Synthetic interferon fragment 1-22 has been analyzed for its potential antigen characteristics. Although no observable anti-interferon titer was found in sheep immunized with 1-22 coupled to multichain polyalanine-lysine labelled 1-22 is precipitated by anti-interferon and the binding can be inhibited by unlabelled crude interferon preparations. This allows the possibility for development of a quantitative radioimmunoassay using synthetic fragments as the labelled antigen.

BALB/c mice were immunized with highly purified interferon preparations and after three secondary boosts the spleen cells were hybridized with a sub line of the BALB/c mouse meyelome MOPC-21 (NS-1). Increased yield of positive responding clones has been attained by in vitro immunization of primary immune spleen cell cultures 3 days prior to hybridization. The hybridization was performed by incubation with polyethylene glycol 1500 and the primary cultures were grown in microtiter trays and assayed for neutralizing activity toward interferon. Fourteen out of 105 initial cultures were positive for anti-interferon production by neutralization assays. Currently, culture conditions have been developed for the maintainence both

in vivo and *in vitro* of mouse meyeloma cell line Sp 2/0-Ag14, a cell line shown to be capable of efficient hybridization with production only of the spleen cell parent immunoglobulin and more of its own meyeloma proteins (Schulman *et al.*, Nature, *276*, 269, 1978). When viable clones are obtained which are positive for anti-interferon they will be grown both as *in vitro* cultures and *in vivo* as peritoneal ascites tumors in BALB/c mice. The yield of IgG from one mouse tumor can be as much as 0.5-1 g of protein. This purified monoclonal immunoglobulin will then be used in interferon purification procedures, radioimmunoassays, and receptor identification investigations.

Publication:

Smith, M.E.: Preparation of Antibodies to Human Lymphoblastoid Interferon. In Pestka, S. (Ed.): *Methods in Enzymology*, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,032-02 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Hemoglobin Synthesis in Cultured Erythroid Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.L. Luskey	Research Associate	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been combined with Z01 AM 25,016 LCB.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,033-02 LCB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Enzymatic Resynthesis of Peptide Bonds in Organic Media

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.A. Homandberg	PHS Research Fellow	LCB NIAMDD
	I.M. Chaiken	Research Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

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Laboratory of Chemical Biology

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INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been combined with Z01 AM 25,003 LCB.

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,034-01 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Processing of Mammalian Sperm Nuclei by Mouse Oocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Dean	Senior Investigator	LCB NIAMDD
	S. Harwood	Physical Science Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.6	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To further our understanding of the molecular mechanism of mammalian fertilization, we are studying the structure of murine sperm chromatin and the molecular processes of nuclear decondensation that occur after sperm penetration of the egg. Using endonucleases as probes, we have documented the resistance of the condensed sperm DNA to nucleolytic digestion. This resistance is markedly diminished after the induction of in vitro decondensation with reducing agents and proteolytic enzymes. Using our ability to decondense sperm chromatin in vitro we are assaying for oocyte macromolecules that mediate the decondensation reaction.

Project Description:

Objectives: Our laboratory is interested in the molecular events of mammalian fertilization as a model system to study developmental biology. During spermatogenesis the somatic histones of the sperm nuclei are replaced by protamines rich in cysteines which, presumably form multiple disulfide bonds, and account for the highly condensed morphologic structure of the chromatin. After penetration through the outer vestments of the egg and the oocyte plasma membrane, the sperm's dense chromatin undergoes dramatic decondensation in the egg's cytoplasm. Subsequently it forms the male pronucleus which fuses with the female pronucleus prior to the first cell division. Our objective is to better understand the structure of sperm chromatin and to study the molecular events that cause the intracellular decondensation of the sperm nucleus.

Methods and Major Results: The caudal head of epipdymi from NIH random bred male mice are minced in tissue culture plates to release mature motile sperm. These sperm are sieved through a nylon screen, washed and incubated with dithiothreitol and cetyl trimethylammonium bromide to release surrounding membranes and tail structures. The resulting products appear as morphologically dense helmet shaped nuclei free of any contaminating tail or membrane structures.

The DNA of these nuclei is extremely resistant to cleavage by endonucleases. Using published procedures, nuclei obtained from mouse spleen lymphocytes were readily digested with either staphylococcal nuclease or with DNAase I. Staph. nuclease digestion of lympohcyte nuclei yielded the typical nucleosomal DNA pattern on 1.2% agarose gels stained with ethidium bromide. DNase I digestion of the control lymphocyte nuclei cut within the nucleosomes yielding oligomers of DNA that were the typical multiples of 10 bases. However, digestion of the sperm nuclei by either enzyme under similar conditions or under conditions of 100 fold excess of enzyme concentration and/or 20 fold increase in digestion time yielded no such products. There appeared to be little if any digestion of high molecular weight sperm DNA into smaller fragments. Nor did the morphology of these nuclei change after such digestions.

By morphologic criteria the sperm nuclei are also resistant to degradation by trypsin. However, in the presence of dithiothreitol, trypsin caused nuclear decondensation as has been reported by others. Radioactively labeled sperm nuclei are obtained by injecting mice intraperitoneally with ^{3}H-thymidine and sacrificing these animals after thirty days to isolate their sperm nuclei. By preincubating the DNA labeled nuclei with trypsin in the presence of dithiothreitol and quenching the reaction with trypsin inhibitor, the labeled DNA becomes susceptible to digestion with DNase I. We are now in the process of establishing the molecular properties of the digestion products in hopes of better understanding the structure of sperm chromatin.

Significance to Biomedical Research and Proposed Course of Project: Greater understanding of the molecular biology of fertilization will signifi-

cantly affect our ability to modulate sperm-egg interactions both to overcome infertility and possibly to introduce effective means of contraception. More specifically, the molecular mechanism of chromatin decondensation and the reconstitution of the DNA onto somatic histones probably involves processes that are also operative in somatic cell mitosis. Thus, an understanding of these processes in fertilization may also be generalized and enhance our knowledge of somatic cell division.

Our immediate future goal is to gain a more complete understanding of the structure of the sperm chromatin and the interaction of the protamines with the DNA using endonuclease digestion as a probe. We then plan to use this knowledge to devise an assay of sperm nuclear decondensation and go on to identify those components in the egg cytoplasm responsible for this process.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,035-01 LCB

PERIOD COVERED
July 1979 - June 1980

TITLE OF PROJECT (80 characters or less)

Hemoglobin Binding to Erythrocyte Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Arthur B. Schneider	Guest Worker	LCB NIAMDD
	Ann Eastlake Dean	Chemist	LCB NIAMDD
	Alan N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been proposed that the interaction of hemoglobin with erythrocyte membranes has important physiological and pathological implications. An equilibrium competitive binding assay has been developed using reductive methylation to tritiate hemoglobin. We have found that in vitro binding is only detectable under non-physiological conditions, i.e. very low pH or ionic strength. It is possible that under the non-ideality conditions in the erythrocyte that some interaction occurs; however, experimental or theoretical demonstration of this remains to be accomplished.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: This project is designed to investigate the binding of hemoglobin to the erythrocyte membrane, a phenomenon recently reported by several laboratories. Our goals are to devise a convenient method for measuring this binding, to investigate its characteristics, and to determine if it is of potential biologic or pathologic significance.

Methods Employed: The methods of competitive binding assays using radiolabelled ligands were used. [^{3}H] was incorporated into hemoglobin by reductive methylation using [^{3}H]-$NaBH_4$. Standard methods for preparing unresealed erythrocyte ghosts and for separating bound and free radioligand were adapted for these experiments.

Major Findings: The binding of ^{3}H-HbA was studied in 5 mM phosphate, pH 6.2. Increasing pH or increasing ionic strength drastically reduced binding. By pH 7.0 or 0.07 M NaCl binding was undetectable (<2%). Even at 0.01 M NaCl the affinity of the sites with the highest binding constant was reduced 20-fold. The displacement data, plotted by the method of Scatchard, were curved, with $K_d = 2 \times 10^{-7}$ and 1.32×10^{6} sites/ghosts for the high affinity sites. Displacement curves indicated the following order of binding: HbS>HbA>HbF, with a 4-fold difference between successive hemoglobins. The order and relative potencies were the same using ^{3}H-HbS, indicating that the hemoglobins bind to the same sites. Glyceraldehyde 3-phosphate dehydrogenase was a more potent competitor than the hemoglobins.

These data do not support a physiologic significance for hemoglobin binding to erythrocyte membranes. However, due to the uncertain nature of the activity coefficients in the erythrocyte it is not possible yet to be certain that a significant interaction does not occur. Although the phenomenon shows specificity and a significant binding constant, this is only true in vitro under non-physiologic conditions. The binding appears to occur to the band 3 sites as well as some others.

Significance: It has been proposed that hemoglobin binding to the erythrocyte membrane could serve as an initiator of hemoglobin S gelation. It is also possible that some forms of metabolic control could be mediated by hemoglobin-membrane interactions. Although the present data do not support these possibilities, they remain to be tested more directly. Theoretical analyses of possible binding due to non-ideality also require further consideration.

Proposed Course: Terminated July, 1980.

Publication:

Schneider, A.B. and Schechter, A.N.: Isoelectric focusing and electrophoretic studies of human hemoglobins. In Deyl, Z. (Ed.), Electrophoresis A Survey of Techniques and Applications (Part B), Amsterdam, Elsevier, North-Holland, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,036-01 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Osmotic Stress Studies of Sickle Hemoglobin Polymerization

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Prouty	Guest Worker	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)
Physical Sciences Laboratory, Division of Computer Research and Technology (Dr. Adrian V. Parsegian)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Polymerization of deoxy sickle cell hemoglobin molecules results in the formation of a gel phase. This gel appears to be polymorphic - in the structure of the fibers formed, and in the packing and alignment of the fibers into aggregates. To study the phase transition from solution to gel in hemoglobin and the gel structure, we have applied an osmotic stress technique. We have developed an expression for the expected behavior under osmotic stress of a solution of a non-aggregating protein and demonstrated its validity for insulin. Solution samples stressed at any osmotic pressure greater than that of a saturated solution were converted to insulin crystals with complete removal of solvent.

Experimental methods and equilibrium tests were developed which permit osmotic stress measurements of the solution-gel transition of deoxy sickle hemoglobin. A gel phase is observed when HbS solutions are concentrated to saturation by stressing; at greater stresses the concentration of the gel phase continued to increase gradually with stress. These results indicate a complex system in accord with other observations of polymorphism. Theoretical models for polymerization and gel structure are being tested using formulations applicable to stress data.

Project Description:

Objectives: Osmotic stress is the force which a concentrated solution of an inert polymer (such as dextran) exerts in drawing solvent from a solution or suspension equilibrated across a semi-permeable membrane. As the dextran concentration is increased, the water in the system under stress comes to equilibrium at a lower chemical activity; the change representing the work necessary to remove a particular portion of the water present in the stressed system. Correlations can be made with the forces (electrostatic, hydrophobic, etc.), among solute molecules and between solute and solvent calculated from independent structural data and models. Using such calculations, and measurements of interlamellar distances by X-ray diffraction, it has been possible to quantify the effects of intermolecular forces in phospholipid bilayers and in TMV and muscle suspensions. It was found also that osmotic stress can bring about phase transitions in lipid bilayers under controlled conditions.

We have begun a study of the potential of this method to study the mechanism and structure of deoxy sickle hemoglobin gelation. Because osmotic stress had not previously been used for proteins, our first objective was to predict its effect on those protein solutions which show a simple transition to crystalline precipitate when their solubility is exceeded. When solutions of insulin, a non-aggregating protein, are exposed across a cellulose membrane to dextran solutions whose osmotic pressure just barely exceeds that of the saturated protein solution, precipitation of crystalline protein should occur. Behavior under osmotic stress cannot be so easily predicted for proteins which aggregate or polymerize in concentrated solution. Deoxy sickle hemoglobin gels have been found to be polymorphic in the degree of alignment of fibers, the interfiber distance, and in the arrangement of individual HbS chains. Fibers convert to crystalline forms under various conditions of preparation. Osmotic stress measurements, which give chemical potentials directly, can provide a unique means of testing models of aggregation and gelation developed from polymer solution theory.

In the initial stage, our study has included objectives which did not require the determination of structural parameters by X-ray or electron microscopy methods. We have attempted to find out 1) how gels could be made by osmotic stress under deoxy conditions; 2) whether values of solubility of deoxy HbS could be obtained from data on concentrations of Hb and osmotic stress at gelation and how these values compared with those from other experiments; 3) whether such solubility data, obtained at a number of temperatures, pH's etc., could be treated by standard thermodynamic means to yield values of ΔH, ΔS, etc. for the solution-gel phase transition; 4) whether stressed gels have the same structure as gels prepared by other methods; and 5) whether examination of Hb concentration for each applied osmotic pressure can yield information helpful in deciding among proposals for the structure of the gel.

An additional objective has been the development of suitable experimental conditions for making stressed gels as samples suitable for measurements by ^{13}C NMR, X-ray diffraction and optical methods.

Methods: Sickle hemoglobin and hemoglobin A were prepared by standard methods. Dextran T500 (Pharmacia) was made up into stock solutions in the appropriate buffer (0.15 M phosphate for Hb, 0.01 M sodium phosphate, 0.1 M NaCl for insulin (Sigma)) by weight. An osmotic cell was made by suspending a small sac made from cellulose dialysis tubing (MW cutoff 12-14,000) containing hemoglobin solution in a test tube of dextran solution. The cell was assembled and sealed under N_2 using dextran solutions which had been degassed; both Hb and dextran were made 0.05 M in sodium dithionite. Contents of the sac and the test tube were under equal pressure of N_2.

An initial experiment was performed to ascertain that final protein concentration was a function only of the osmotic pressure of the stressing solution and was independent of initial concentration of protein. This experiment also served to establish a time frame for equilibration (less than 3 days at 3°C). It was taken as proof that equilibrium had been reached that final concentrations were independent of attainment by concentration or dilution or by heating or cooling. They were also independent of whether the initial state was gel or solution.

Concentration of hemoglobin in solutions were measured by removing aliquots with a 10λ Hamilton syringe, converting the sample to cyanomethemoglobin at appropriate dilution, and measuring abosrbance at 540 nm. Concentration of samples which had gelled were measured by melting the gel at 0°C or by dissolving overnight in a measured amounts of a concentrated KCN solution, and the concentration was then measured as cyanomethemoglobin. Both gel methods involved terminating the experiment with the sample used; a "carousel" was devised which permits dividing one Hb sample into six small sacs which can be removed successively under deoxy conditions. Alternatively, the carousel permits simultaneous equilibration of different hemoglobins (e.g. A&S) against the same dextran solution.

Major Results: We have shown that the osmotic stress technique permits us to distinguish between the simple phase transition represented by the crystalline solubility of insulin and the more complex change of state observed in the aggregation and gelation of deoxy sickle hemoglobin. For insulin a dramatic change occurred: all solutions equilibrated against dextran solutions of greater than a critical concentration showing complete loss of solvent - only insulin powder remaining on the protein side of the membrane.

Deoxy HbS solutions which are concentrated by osmotic stress until they do not flow in the dialysis sac (osmotic cell) are described as having "gelled". We have found a small range of hemoglobin concentrations (and corresponding dextran solutions) over which the solution to gel transition occurs. Tests using a number of closely-spaced dextran concentrations are being carried out to determine gelation as a function of stress. When total hemoglobin concentration is plotted against applied stress, no abrupt change in slope is seen at the observed solution-gel transition, although such behavior does occur at somewhat higher concentrations. As stress on the gelled system increases, a gradual increase in Hb concentration occurs, indicating continual removal of buffer. This result differs from that seen

in insulin, and is not consistent with a gel which is made up, at all total hemoglobin concentrations, of a saturated solution of hemoglobin (Csat) in equilibrium with increasing amounts of polymer. We are analyzing concentration data so as to permit us to distinguish between a theoretical long-lasting "metastable" intermediate and the existence of equilibrium "tactoidal" polymeric gel phases, at each particular hemoglobin concentration.

Solutions of oxy HbS and deoxy and oxy HbA show large increases in viscosity as hemoglobin concentration increases under osmotic stress; however, at any particular stress, hemoglobin concentration and viscosity are lower for these samples than for deoxy HbS. More data are being obtained to permit analysis of these relationships.

Significance: The criteria for therapeutic agents in sickle cell anemia, on the molecular scale, have been increase of deoxy HbS solubility to prevent gelation and/or increase in the delay time for gel formation to prevent sickling in capillaries. The proposal of a tactoidal phase implies that the degree of alignment (or the existence of metastable states) may be of some importance in pathogenesis. In the simplest case, rods such as those of deoxy HbS fibers would be expected to show greater alignment in the absence of repulsive interactions or of a "metastable" state. The possibility exists, then, that inducing erythrocyte changes which decrease rod-rod alignment or increase inter-rod repulsions may be beneficial. Systematic studies of gel structure at the level of packing and alignment of fibers as a function of gelation conditions may provide a test of this premise. Identification of the repulsive forces or interactions between deoxy HbS fibers provide information about alignment processes.

Proposed Course: The simple experiments undertaken initially have provided enough data to confirm the validity of the osmotic stress technique in the study of protein solubility and aggregation. Complete data for a model crystallizing protein will be obtained. The immediate goal is to complete the series of time, temperature and pH dependent experiments relating total hemoglobin concentration and gel formation to the applied osmotic pressure from dextran solutions for both HbA and HbS in the oxy and deoxy states. Such thermodynamic parameters as solubility, ΔS and ΔH will be calculated and compared with published results. We will continue to work toward an accurate assay for Hb concentration in dense gels (C_{Hb} > 40 g/dl). A means of preparing stressed gel samples suitable for NMR measurements is being developed and from ^{13}C NMR measurements we expect to find out whether stressed gels contain monomeric HbS (or aggregates of < 10 HbS) and in what proportion to polymer.

The theoretical treatment of phase transitions in self-assembling bimolecular systems has been the subject of several recent papers. The presence of monomers and of polymer molecules of varying and non-fixed length suggests phase changes (as total hemoglobin concentration increases) from an isotropic solution phase, through a two-phase equilibrium of monomer solution with polymer, to a single "tactoidal" phase. This phase is anisotropic and alignment increases continually as polymer concentration increases in the

phase. Osmotic stress data, which yield chemical potentials and can be used to obtain information on the order of phase transitions, provide a unique test for these theories. We shall continue to develop formulations to analyze results in these terms.

Study of the gel structure as a function of conditions of preparation will require measurement of a structural parameter which may be expected to change with stress (packing and/or alignment of deoxy HbS fibers). Preparation of stressed gel samples suitable for fiber X-ray diffraction, for birefringence and possibly for electron microscopy measurements, will be undertaken. We shall also use water proton magnetic resonance measurements as an indicator of changes in water content and of "binding" of water to hemoglobin.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,037-01 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Functional Domains of the Hemoglobin Molecule and Globin Gene Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Fred K. Friedman	Staff Fellow	LCB NIAMDD
	Alan N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

Laboratory of Physical Chemistry, NIAMDD (Dr. William A. Eaton)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The presence of non-expressed intervening sequences of DNA (introns.) in globin genes has led to suggestions that each expressed sequence codes for a domain of specific function within the protein. We plan to explore this possibility by preparing the putative domains by selective cleavage of the hemoglobin chains and characterizing each resultant peptide with reference to the known function of its amino acid residues in the intact molecule. Initial experiments with the intact chains have established improved procedures for the preparation and handling of separated globin chains, their reconstitution to hemoglobin, and their cleavage to peptide fragments.

Project Description:

Objectives: Various studies on protein fragment systems have provided much information on the factors governing protein structure and function. A specific example of this type is the preparation of fragments from proteins which consist of several easily distinguishable structural domains. An individual domain often retains the same specific properties it exhibits in the intact molecule, such as binding to a particular ligand or enzymatic activity; but different domains may modify each others' behavior when covalently limited.

The recent discovery that in eukaryotic genes expressed nucleic acid sequences (exons) are separated by intervening sequences (introns) has led to proposals that exons code for specific functional units within a protein. These units may be identified with the domains studied by protein chemists. Recombination of adjacent exons would therefore result in different interdomain interactions in the coded protein and alter its efficiency in carrying out its biological function.

The relationship between the DNA exon and protein domain is most evident in the immunoglobulins. This relationship may not be as evident in other proteins because domains may engage in long range interactions to such an extent as to preclude easy recognition of the interdomain interfaces. However, knowledge of intron locations would readily suggest cleavage points on the polypeptide chain for generating domains. Studies with these fragments should in turn provide further information on the link between gene and protein structure.

Since the hemoglobin molecule is so well characterized and its α and β globin genes sequenced, it is well suited for the type of studies mentioned above. Three exons code for each chain, and known functions can be localized within each of the corresponding domains. For example, the central fragment binds heme and contains the $\alpha_1\beta_2$ contact residues, the C-terminal fragment contain the $\alpha_1\beta_1$ contacts, and various regulators bind to both terminal fragments.

Our goal is to prepare all three fragments and to determine the structural and functional characteristics of each fragment individually and in combination with other fragments. Particular attention will be focused on the interaction of heme with the central domain and of its ligand binding capability in the absence and presence of neighboring domains and allosteric effectors.

Methods: Since arginine residues are coded for at or near the exon-intron interfaces, treatment with the arginine-specific protease clostripain should cleave the chains at the desired positions. After establishment of optimal conditions for digestion, the peptides of interest are purified and characterized by standard techniques of protein and peptide chemistry.

Results: The separted heme-containing subunits (α^h and β^h, respectively) were found to be resistant to proteolytic digestion under a number of experimental conditions. The reaction was therefore carried out on the relatively unstructured heme-deficient subunits (α^o and β^o, respectively).

Cleavage into smaller peptides proceeded readily and was ascertained by sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis of the digests. Partial purification of the β^o fragments was achieved by gel chromatography under acidic conditions.

While proceeding with peptide purification, we realized that the literature on the preparation and properties of α^o and β^o and of their reconstitution with hemin to hemoglobin is inadequate for our purposes. Additional methodology and experience with the entire polypeptide chains was necessary before additional work with their fragments could be carried out.

The current procedure for preparation of globins is a multi-step procedure requiring a week of work and results in low yields of renaturable material. It involves separation of chains by treatment with p-mercuribenzoate, removal of the covalently bound reagent from each isolated chain, and heme removal. A more rapid procedure was developed which requires several hours and involves an initial removal of heme from whole hemoglobin, and separation of the constituent α^o and β^o chains in a one step column chromatographic procedure.

Optimal conditions for the addition of hemin to globin to produce a molecule with ligand-binding capability was established. Highest yields and avoidance of formation of undesirable side products was achieved by stoichiometric recombination of protein and porphyrin in the reduced state at alkaline pH, and by ensuring that iron was initially in the reduced state. The α^h and β^h thus produced can associate to yield hemoglobin.

Proposed Course of Future Work: The improved methods will be used to prepare and manipulate the fragments more efficiently. Each exon product will be evaluated as to its known function within the intact hemoglobin molecule, both in the presence and absence of other exon products. Particular attention will be placed on structural changes induced by addition of heme, and of various heme ligands. These will be considered in view of what is presently known of the mechanism of the cooperative interactions of hemoglobin.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

I. POLYAMINES

Mutants Defective in Polyamine Biosynthesis

The physiological role of putrescine, spermidine, and spermine is still completely unknown. In order to study this problem, we have constructed mutants of Escherichia coli and of Saccharomyces cerevisiae that have no polyamines.

In E. coli mutants were obtained for each of the relevant biosynthetic enzymes (e.g., ornithine decarboxylase, arginine decarboxylase, agmatine ureohydrolase, and adenosylmethionine decarboxylase, as well as lysine decarboxylase), and the mutations were combined into one strain. This amine-less mutant grew more poorly than the wild type strain but still could act as the host of the bacteriophage T4, T7, and f2. In contrast, bacteriophage λ could not be induced in the amine-less strain. This defect in λ production is being studied in order to define the step that depends on polyamines.

. . . . . Drs. H. Tabor, C. W. Tabor, and E. W. Hafner

Mutants were also obtained in S. cerevisiae that were defective in ornithine decarboxylase, adenosylmethionine decarboxylase, spermidine synthase, or spermine synthase. We showed that strains without amines can not grow. Deficient strains lose double-stranded RNA killer plasmid and cannot sporulate. As part of these studies we have investigated the regulation of ornithine decarboxylase in vivo, and regulatory mutants have been isolated. We have shown that spermine and spermine synthase are very important in the regulation of ornithine decarboxylase; mutants defective in the biosynthesis of spermine have very high levels of ornithine decarboxylase. Since yeast is a eukaryote, these studies have direct relevance to the control of ornithine decarboxylase in normal and neoplastic growth of mammalian cells, a subject which has been of special interest to many laboratories.

. . . . . Drs. M. S. Cohn, C. W. Tabor, and H. Tabor

Enzyme Studies

Adenosylmethionine Synthetase. Adenosylmethionine synthetase is a very important enzyme as it is the first enzyme in the pathways leading to the biosynthesis of polyamines and of all methylation reactions. We have now purified this enzyme to homogeneity from E. coli, and have carried out extensive studies on the kinetics and mechanism of the reaction. Of particular interest is the intermediary role of an enzyme-bound tripolyphosphate, the presence of a concurrent tripolyphosphatase activity, and a requirement for both a monovalent and a divalent cation. The purification was facilitated by isolating a ColE1 plasmid containing the gene for adenosylmethionine synthetase and placing this plasmid in a strain which is derepressed for the synthesis of this enzyme. Sufficient enzyme was easily obtained to permit crystallization and preliminary X-ray crystallography. [The X-ray crystallography was carried out by Drs. Gary L. Gilliland and David R. Davies (LMB, NIAMDD).]

. . . . . Drs. G. D. Markham, C. W. Tabor, and H. Tabor

Adenosylmethionine Decarboxylase. With the help of plasmid-containing strains, adenosylmethionine decarboxylase has been purified and studied. This enzyme is of particular interest since, with Dr. Reed B. Wickner, we have previously shown that it contains covalently-bound pyruvate as an essential moiety.

. . . . . Drs. G. D. Markham, C. W. Tabor, and H. Tabor

Ornithine Decarboxylase. Particularly high concentrations of ornithine decarboxylase can be obtained from a mutant strain of S. cerevisiae that lacks adenosylmethionine decarboxylase. Ornithine decarboxylase is being purified from this source.

. . . . . Drs. C. W. Tabor, H. Tabor, A. K. Tyagi, and M. S. Cohn

II. MECHANISMS OF INHERITANCE IN SACCHAROMYCES CEREVISIAE

The Killer Virus-Plasmid of Yeast

Background. Killer strains of yeast are those secreting one of two protein toxins coded by one of two double-stranded RNAs. M_1 ds RNA encodes the K_1 toxin, and M_2 ds RNA encodes the K_2 toxin. All strains (killer or not) carry another ds RNA, called L, which encodes the major coat protein in which all ds RNA molecules are found as virus-like particles. We have previously described 3 chromosomal genes whose products are essential for expression of information in M_1 ds RNA (kex and rex genes), 29 chromosomal genes needed for maintenance or replication of M_1 but not for L (27 mak genes, pet18, and spe2), and 6 genes with regulatory effects on K_1 plasmid replication (4 ski genes, KRB1, and a mitochondrial gene). We have also discovered a replication gene (sd) located on the killer plasmid M_1 ds RNA. A new plasmid, called [EXL], eliminates M_2 ds RNA when both are introduced into the same cell. A second new plasmid, [NEX], prevents [EXL] from eliminating M_2 ds RNA. Surprisingly, M_2 ds RNA needs a new chromosomal gene, MKT1, only if [NEX] is present.

New Findings. We have now discovered a third new yeast plasmid by its effect on K_1 killer plasmid replication. The replication-defective mutant, [KIL-sd_1], cannot replicate in the usual wild type host. We originally discovered it as a mutant M_1 ds RNA which needs a host ski$^-$ mutation to be maintained. Another yeast plasmid can, however, allow [KIL-sd_1] to replicate even in a SKI$^+$ host. We call this [HOK], for "helper of killer."

[HOK] needs, for its replication, the MAK10 and PET18 gene products, but not 11 other MAK gene products. [NEX] needs only MAK3 of 4 genes tested. [EXL] needs all of the 4 MAK genes tested. None of these 3 new yeast plasmids correspond to ds RNA species detectable by gel electrophoresis of purified ds RNA.

Three genes involved in regulation of acid phosphatase activity have been located on the yeast genetic map.

2 μ DNA Plasmid. Saccharomyces cerevisiae cells carry a 4×10^6 dalton double-stranded circular DNA plasmid called 2 μ DNA. This plasmid's replication resembles chromosome replication, but because it does not give the cells a noticeable phenotype and because previously constructed recombinant plasmids with a 2 μ DNA replicon were unstable, it has not been possible to study the genetics of this replicon. We have constructed a stable plasmid carrying the

yeast LEU2 gene and the 2 μ DNA plasmid replicon. This recombinant plasmid can now be used for genetic studies of 2 μ DNA replication.

Molecular Cloning. We have cloned the yeast URA1 gene coding for dihydro-orotate dehydrogenase. The gene is expressed in Escherichia coli allowing pyrD mutants to grow without added uracil.

. . . . . Drs. R. B. Wickner, P. Guerry-Kopecko, R. M. Sheetz, and S. P. Ridley

III. NUCLEIC ACIDS

Evolution and Regulation of Genes Involved in Protein Synthesis

Evolutionary Studies

(a) A wide variety of completely unrelated prokaryotes share a set of DNA sequences that are highly homologous to the Escherichia coli tuf gene. In E. coli, this gene encodes peptide chain elongation factor Tu that binds amino-acyl tRNA to the A-site of the ribosome.

(b) This type of sequence conservation is unique to the tuf gene, a finding that suggests that the widespread presence of this set of highly conserved tuf-homologous sequences is not due to recent gene transfer among prokaryotes but is due to strong selective pressure. Due to the degeneracy of the genetic code, DNA sequences need not and have not been found to be conserved in order to conserve protein sequence. Therefore, the conservation of tuf-homologous sequences could mean that evolutionary selection can operate at the level of codon utilization.

(c) We have found duplicate tuf genes in every gram-negative organism examined (10 enteric, 5 nonenteric) but only one tuf gene in the two gram-positive organisms that we tested. By correlating the number and chromosomal proximity of the tuf genes with the linkage maps and phylogenetic relationship of the organisms that we have examined, we postulate the following scenario for the evolution of the tuf gene duplication. Approximately one billion years ago when the gram-positive and gram-negative branches diverged, the tuf gene was duplicated in one of the early gram-negative progenitors. The duplicated tuf genes remained in close proximity on the chromosome until the time when the more recently evolved genera arose, such as the enteric family, when one of the tuf genes and a portion of the nearby chromosome was translocated to a distant part of the chromosome.

. . . . . Drs. D. Filer and A. V. Furano

Studies on tuf Gene Regulation

(a) The synthesis of EF-Tu is regulated in part at the level of translation. tuf mRNA (especially that produced from the tufA gene) is translated at least 3 times more efficiently than any other mRNA, including the one for EF-G, and our finding completely explains how the cell can maintain the 8- to 14-fold molar ratio of EF-Tu to the other proteins required for peptide chain elongation.

(b) Part of the EF-Tu mRNA (which is 1,200 bases long) is produced from the tufA gene by a specific endonucleolytic cleavage of the large (4,000 bases long) transcript of the operon which contains the tufA gene. This is the first example of post-transcriptional modification of mRNA molecules in

prokaryotes and may be related to the high translational efficiency of the tufA gene transcript.

(c) The transcriptional activity of the operon that contains the tufA gene (also called the str operon) in some way regulates the activity of all of the rRNA gene operons. We find that cells which cannot decrease transcription of the tufA-operon cannot decrease the synthesis of rRNA even under conditions of cell growth that in normal cells markedly decrease the synthesis of rRNA.

. . . . . Drs. F. S. Young and A. V. Furano

(d) We have recently determined the DNA sequence of the beginning of the tufA gene with the aim of trying to understand the structural basis for the high translational efficiency of the tufA mRNA. Unexpectedly, we found that some of the DNA sequence does not correspond to that which would encode the amino acid sequence of EF-Tu. These findings imply that part of the sequence for this part of the EF-Tu molecule may be derived from elsewhere in the genome. We are currently determining the DNA sequence of the corresponding part of the tufB gene.

. . . . . Dr. A. V. Furano

Mechanism of Action of Nerve Growth Factor and the Regulation of Eukaryotic Genes Involved in Cell Development. We have recently begun a program to identify and isolate those rat genes that must be expressed to attain or maintain a particular state of cellular development or growth. We are using a cloned pheochromocytoma cell line, pC12, that can respond to nerve growth factor by differentiation from a dividing cell to a nongrowing, electrically excitable neuronal-like cell, as well as regenerating rat liver cells as model systems. We are now in the process of generating three genomic libraries from rat DNA: one comprising the entire genome, one comprising just the repetitive DNA fraction, and one consisting of the repetitive DNA and the single copy DNA that is contiguous with the repetive DNA. So far we have found that there are differences between the small nuclear RNA population of nondividing and dividing hepatocytes as well as between these RNA's and those found in the pC12 line. We have also found that significantly different and nonoverlapping regions of the rat genome are expressed in rat liver cell and the pC12 cells.

. . . . . Drs. A. V. Furano and F. R. Witney

DNA Replication

We are using bacteriophage T4 proteins to study the reactions required for the replication of duplex DNA. We have previously shown that the T4 gene 41 protein and a protein controlled by T4 gene 61 synthesize a heterogeneous family of pentaribonucleotides with the sequence pppACNNN on single-stranded DNA templates. These oligonucleotides serve as primers for DNA synthesis and are elongated by the T4 DNA polymerase (gene 43 protein) in conjunction with the T4 45 protein and the complex of the T4 gene 44 and 62 proteins (DNA-dependent ATPase). Strand displacement synthesis by the addition of nucleotides to a 3' OH at a nick in duplex DNA is accompanied by the unwinding of the complementary strand and requires the T4 gene 32 protein (helix-destabilizing protein), in addition to the 43, 45, and 44/62 proteins.

During the past year we have extensively purified the priming activity controlled by gene 61 and have shown that it copurifies with a protein with a

denatured molecular weight of 42,000. The 61 activity also has a native molecular weight of about 45,000 and behaves as a basic protein during electrophoresis at pH 7.8. A 42,000 molecular weight protein, labeled by the addition of [^{14}C]amino acids following T4 infection, cochromatographed with the enzymatic activity during DNA cellulose fractionation of 61^+ extracts and was missing from 61^- extracts. This suggests that the priming activity is due to a phage protein rather than to a host protein modified by the gene 61 product. The 42,000 molecular weight protein comprises only about 80% of our purest preparations. Therefore it is possible that more than one protein is required for the 61 activity.

We have been unable to separate the roles of 41 and 61 proteins in primer synthesis, since no partial reactions have been found for either protein. 41 protein does copurify with a single-stranded DNA-dependent nucleotidase. Primer synthesis by the 41 and 61 proteins is greatly stimulated by 32 protein when 61 protein is present at high concentration. The same pentanucleotide products are made in the presence and absence of the 32 protein.

We are searching for factors required for the initiation of new chains within duplex DNA templates, since this reaction cannot be catalyzed by the seven T4 proteins isolated so far. We have shown that concomitant RNA transcription by Escherichia coli RNA polymerase is not required for the initiation of T4 DNA synthesis in vivo. Since E. coli RNA polymerase is required for the synthesis of T4-induced enzymes which are involved in DNA replication, we have temporally separated the synthesis of early enzymes from DNA replication by infecting at a nonpermissive temperature with reversible temperature-sensitive mutants in gene 43 or 45. After a time at high temperature sufficient to make the early proteins, the temperature is lowered to allow DNA synthesis to begin. The addition of the RNA polymerase inhibitor rifampicin at the time of the shift to the lower temperature does not change the kinetics of T4 DNA replication. In vitro, synthesis on duplex T4 DNA with the seven T4 proteins is not increased by purified E. coli RNA polymerase, T4 modified host RNA polymerase, or the T4 gene 39 and 52 encoded ATP-dependent nicking-closing enzyme. These negative results do not exclude the possibility that one or more of these proteins is involved in DNA initiation, since origin recognition may require additional proteins and/or a topological configuration of T4 DNA not present in our soluble systems. We are therefore characterizing the DNA made in extracts of gently lyzed infected cells to see if there is preferential synthesis of any portion of the T4 genome. DNA synthesis in these extracts is completely dependent on T4 proteins, since it does not occur in extracts infected with 41^- or 45^- mutants, unless they are supplemented with the corresponding purified protein. Synthesis in a 41^- extract, supplemented with 41 protein, is dependent on the addition of exogenous DNA and shows some preference for T4 DNA.

In the course of studies characterizing the DNA molecules made in crude extracts, we have found a T4 nuclease which produces products giving discrete bands on agarose gel electrophoresis. This nuclease activity is not present after infection with a T4 $33^-/55^-$ mutant, which is defective in late protein synthesis. It is present after infection with wild type phage, as well as mutants in several genes whose phenotypes suggest they may control a nuclease (46, 47, 49, den A, den B, rII). The nuclease degrades T7 and λ DNA but has little or no activity on T4 DNA. The pattern of product bands is too complex for a

simple restriction enzyme. We are currently purifying this activity to see whether it is a mixture of sequence-specific restriction nucleases, or is a single protein whose cleavage is determined by some other structural feature of the DNA.

. . . . . Drs. L. L. Silver, M. Venkatesan, and N. G. Nossal

Evolution

The capacity of nucleotide bases to form hydrogen bonds with peptides has been further investigated, and identification made of the interacting groups of peptides and cytosine, which bind very strongly.

. . . . . Drs. C. H. Niu and S. Black

IV. COMPLEX CARBOHYDRATES

Gangliosides that bind cholera toxin can be detected by the direct binding of ^{125}I-labeled toxin to thin-layer chromatograms followed by autoradiography. The method is very sensitive: 0.1 ng of G_{M1} (70 femtomoles) can be detected on chromatograms of total lipid extracts of cells. The methods may be useful in defining the receptors of other toxins or proteins such as some lectins, antibodies, and hormones that bind carbohydrates.

Anti-lacto-N-difucohexaose I IgG purified by affinity chromatography from the serum of a goat immunized with lacto-N-difucohexaose I coupled to polylysine is specific for the Le^b antigen but its usefulness for the routine determination of Lewis blood type is limited because the antibody does not agglutinate human erythrocytes. However, the antibody can be converted into a powerful Le^b agglutinin by polymerization with dimethyl suberimidate.

A recently-developed radioimmunoassay for Le^b glycolipids in human plasma was used in order to explain why pregnant women tend to lose their Lewis blood type. The results obtained suggest that a redistribution of glycolipids from erythrocytes to serum lipoproteins during pregnancy accounts for this phenomenon.

. . . . . Drs. D. F. Smith, T. E. Rohr, C. V. Warner, J. L. Magnani, and V. Ginsburg

V. MEMBRANE STUDIES OF ESCHERICHIA COLI AND OF MACROPHAGES

The Outer Membrane of E. coli

The outer membrane permits small hydrophilic molecules to enter through pores but excludes hydrophobic molecules, including antibiotics, drugs, and detergents. Its protein, lipid, and lipopolysaccharide (LPS) composition is known, but their spatial arrangement, the precise structure of LPS, and the mechanism of the exclusion of hydrophobic molecules, is still under investigation.

We have previously isolated the first known mutant that, along with increased permeability to hydrophobic compounds, shows changes solely in LPS. We now show that a hydrophobic tetracycline derivative fluoresces more when added to cells of the mutant than when added to the wild type, suggesting that the mutation permits increased membrane association of this drug. When added to purified LPS, this drug also fluoresces more than when added to wild type LPS. This result supports the hypothesis that the change in LPS directly causes the

permeability change by permitting greater partition of this molecule into the LPS of the outer membrane.

. . . . . Dr. L. Leive

Previous studies in this and other laboratories suggested that the polysaccharide of LPS may contain one to at most four size classes in a given strain. Using sodium dodecylsulfate gel electrophoresis, we now can detect up to 40 different lengths of LPS in E. coli and Salmonella typhimurium strains thought previously to have one or two. Each consecutive band represents addition of one or more repeating pentasaccharide antigenic side chain unit to the lipid-core region, as shown by measuring the ratio of radioactivity in side chains to radioactivity in phosphate of the core and lipid.

. . . . . Drs. R. C. Goldman and L. Leive

We earlier devised a method for extracting LPS from whole cells with aqueous butanol resulting in a product that was 90-98% LPS plus 2-10% protein. This material received a great deal of attention from other, immunological, laboratories, as the small amount of protein that copurifies and is pronase-resistant is mitogenic for mutant mice that do not respond to LPS as a mitogen. We have now shown that the proteins that copurify are specifically outer membrane proteins and have proven, with the aid of specific antibody, the identity of two of the four pronase-resistant proteins.

. . . . . Drs. R. C. Goldman and L. Leive

Macrophage Mutants and Cell Function

We have selected mutants of a macrophage cell line that do not show generalized phagocytosis, using their ability to survive addition to the medium of beads coupled to toxic molecules as a selective mechanism. Two stable mutants have been selected so far. These mutants will be studied to determine pathways involved in phagocytosis.

. . . . . Drs. S. Takasaki and L. Leive

We have labeled membranes of macrophage cell lines by borotritide reduction of oxidized sialic acid residues, and defined several labeled protein bands, on sodium dodecylsulfate gels, that completely lose their label on sialidase treatment. These proteins are being used as markers in studies of the specificity of internalization, and rate of entry and recycling, of membrane markers during phagocytosis and pinocytosis.

. . . . . Drs. R. C. Goldman and L. Leive

Derivatives of S. typhimurium, differing only in their LPS side chain composition, were shown by Dr. Makela to vary one thousand-fold in their lethality for mice. In vivo clearance data suggested a role for phagocytosis in this difference. We now show that macrophage cell lines phagocytose the least toxic organism much more rapidly than the most toxic one. The possible role of the macrophage mannose receptor and other receptors in this process is being explored.

. . . . . Y. Takasaki (Guest Worker, LBP), Dr. L. Leive, and Dr. P. H. Makela (Central Public Health Laboratory, Helsinki, Finland)

Aldoheptose Biosynthesis and Its Regulation

A mutant of Escherichia coli K-12 carrying a mutation, designated rfaD, which affects the synthesis of the aldoheptose, L-glycero-D-mannoheptose, has been isolated. It was observed that the lipopolysaccharide of the rfaD mutant contained the aldoheptose D-glycero-D-mannoheptose rather than the normal L-glycero-D-mannoheptose. It is postulated that L-glycero-D-mannoheptose is formed from D-glycero-D-mannoheptose via a racemization. A crude assay to measure the epimerase activity has been developed. Hybrid colicin E1 plasmids capable of correcting the rfaD phenotype have been selected, and these increase the epimerase activity 6- to 10-fold relative to the wild type. This represents the initial step in understanding the biochemistry of aldoheptose biosynthesis and provides a system for studying its regulation.

. . . . . Dr. W. G. Coleman, Jr.

We previously selected from the Clark and Carbon colony bank, hybrid ColE1 plasmids which corrected the rfaD phenotype and conferred ColE1 resistance by F-factor--mediated transfer. This was further demonstrated by transformation experiments with purified plasmids as DNA sources into hsd rfaD strains. We are now characterizing the hybrid ColE1 plasmids and cloning and fine mapping the rfaD gene.

. . . . . Drs. W. G. Coleman, Jr., and Ellis L. Kline

VI. ENZYME MECHANISMS AND PROTEIN STRUCTURE

Tryptophan Synthase of Escherichia coli

The chemistry of the active site of the β_2 subunit of tryptophan synthase is being probed by sequence analysis of specifically modified enzymes. We have reported the primary sequence of residues 1-99 of the β_2 subunit. Three known active site residues were shown to occur in this sequence: lysine-87, which forms a Schiff's base with pyridoxal phosphate; histidine-82 or histidine-86, which removes the α-proton of L-serine; and cysteine-62, which reacts with an affinity reagent, bromoacetylpyridoxamine phosphate. We have subsequently located two other reactive sulfhydryl residues in the primary sequence of the β_2 subunit: cysteine-170, which reacts with N-ethylmaleimide in the presence of pyridoxal phosphate, and cysteine-230, which reacts with N-ethylmaleimide or with 2-nitro-5-thio-cyanobenzoic acid after removal of pyridoxal phosphate. Cysteine-230 is essential for catalytic activity.

The mechanism of action of tryptophan synthase has been investigated by spectroscopic studies of the interaction of the chromophoric pyridoxal phosphate cofactor with substrates and products. Recent studies with L-tryptophan, D-tryptophan, and S-methyl-L-cysteine have led to the identification of chromophoric enzyme-substrate intermediates. An enzyme-substrate intermediate formed from L-serine has been reduced with sodium borohyride and has been isolated for stereochemical studies.

. . . . . Dr. E. W. Miles

Effect of Excluded Volume on Biochemical Equilibria and Kinetics

A statistical-thermodynamic study of the effect of excluded volume upon biochemical equilibria and kinetics has been carried out. Several previously

unobserved but potentially significant phenomena are predicted to occur in solution as the volume fraction of globular macromolecules increases toward levels found in physiological media. Foremost among these are the great enhancement of protein self- and hetero-association processes, and substantial changes in the rates of enzyme-catalyzed reactions.

. . . . . Dr. A. P. Minton

Excluded-Volume-Induced Self-Association of Myoglobin at High Concentration

The self-association of myoglobin at high concentration has been studied by measurement of sedimentation equilibrium of myoglobin and hemoglobin in especially constructed centrifuge cells. Preliminary analysis of the data suggests that self-association of the kind predicted by the aforementioned theoretical study is occurring.

. . . . . Drs. M. S. Lewis (BEIB, DRS) and A. P. Minton

Excluded-Volume-Induced Self-Association of Myoglobin at Low Concentration in the Presence of Other Globular Proteins at High Concentration

The effect of high concentrations of other globular proteins upon the state of aggregation of myoglobin at low concentration has been studied by measurement of the fluorescence polarization of fluorescent derivatives of myoglobin and hemoglobin in the presence and absence of other proteins. The results suggest that proteins having a molecular volume comparable to that of myoglobin induce self-association of myoglobin (probably to a hemoglobin-like tetramer), while larger proteins and random coil polymers do not. These findings are in qualitative accord with predications of the aforementioned theoretical study.

. . . . . Drs. J. Wilf and A. P. Minton

Exclusion Chromatography as a Tool for Studying Self-Association at High Protein Concentration

The dependence of the partition coefficient in exclusion chromatography upon concentration of macromolecular solute has been theoretically studied for highly non-ideal solutions of both nonassociationg and self-associating solutes. It was found that exclusion chromatographic data which were interpreted by other investigators as providing evidence for the self-association of hemoglobin at high concentrations may be quantitatively accounted for by the new theory without invoking self-association of hemoglobin.

. . . . . Dr. A. P. Minton

Aminoacyl tRNA Synthetases

Over a period of years we have accumulated evidence that the glycyl and valyl tRNA synthetases of yeast can exist in two forms. These are present in the cells and vary in concentration in a reciprocal manner during growth. One form of the valyl tRNA synthetase can now be dissociated with separation of a heat-stable, nondialyzable acidic substance that stimulates the enzyme's action.

. . . . . Dr. S. Black

Protein Sequences and Structure

Binding Studies and Protein Interactions. Model systems are being developed and studied for explaining cooperative effects in biological macromolecules, such as hemoglobin, collagen, clathrin, and calmodulin.

Slow Degradation of Proteins. Sterile solutions of proteins yield peptides and amino acids on standing for various periods of time. A number of proteins are being studied in an attempt to explain this phenomenon adequately. It is proposed that some of the observed activity is intrinsic in the protein molecule itself.

Statistical Analyses of Protein Sequences. The concept of uniqueness and a quantitative method for its evaluation has been developed using Monte Carlo statistics. Analyses based on this concept are being used to relate isolated structures with possible biological activity.

. . . . . Dr. H. A. Saroff

Oxidation of Oxyhemoglobin

By measurements near the isosbestic points for oxidation of oxyhemoglobin, it was shown that ferricyanide oxidizes the α and β subunits at about the same rate. Also, it was found that if the β-93 SH groups are converted to thio esters or reacted with inorganic or organic mercury, then the β subunits are oxidized by copper at the same slow rate as the α subunits (in contrast to the fast oxidation of the β subunits in the unmodified protein).

The electron spin resonance spectrum [studied in collaboration with Dr. Hideo Kon (LCP, NIAMDD)] of ferrihemoglobin produced by reaction with hydroxylamine, shows peaks characteristic of denatured nitric oxide hemoglobin, as well as several unidentified peaks. Some of the unidentified peaks are at a field strength close to those for the peaks of heme complexes with sulfhydryl and imidazole, suggesting that the position occupied by a water molecule or hydroxide in ordinary ferric hemoglobin is, in the hydroxylamine-treated compound, occupied by a sulfhydryl.

. . . . . Drs. R. B. Simpson and H. Kon (LCP, NIAMDD)

VII. HORMONES AND RECEPTORS

Insulin Receptors in Genetic Disorders of Insulin Resistance

We have continued to study genetic disorders of peripheral resistance to the polypeptide hormones, insulin and somatomedin (insulin-like growth factor), using fibroblasts established in culture from skin biopsies from affected patients and control subjects. We have characterized specific cell surface receptors on fibroblasts for insulin and somatomedins, as well as the biological responses (glucose incorporation, amino acid transport) to these peptides. We have identified the first selective genetic defect of insulin receptors in fibroblasts cultured from a patient with leprechaunism, intrauterine growth retardation, and clinical insulin resistance. We have examined the effect of this receptor defect on biological functions of insulin. These studies promise to contribute to understanding the relationship of hormone binding to transmission of the biological signal, and to defining the hormonal

regulators of intrauterine growth.

. . . . . Dr. M. M. Rechler, E. E. Schilling, J. A. Romanus, Dr. A. B. Knight

Multiplication Stimulating Activity (MSA): An Insulin-Like Growth Factor (Somatomedin) from Cloned Rat Liver Cells

In our studies of the insulin-like growth factor (somatomedin) multiplication stimulating activity (MSA), a polypeptide synthesized by a cloned line of rat liver cells (BRL 3A), progress has been made in the following areas: (i) characterization of a polypeptide biochemically and immunologically related to BRL-MSA and synthesized by explants of fetal rat liver; (ii) demonstration of 20- to 100-fold increased levels of MSA in fetal rat serum; (iii) characterization of growth hormone-dependent binding proteins for somatomedins in serum of rats and humans; (iv) recognition that insulin and MSA stimulate common biological responses via common pathways but using separate receptors in fibroblasts, adipocytes, liver cells, and cartilage; and (v) demonstration of extraordinary diversity of receptors for somatomedins.

. . . . . Dr. M. M. Rechler, E. E. Schilling, J. A. Romanus, Y. W.-H. Yang, and Dr. A. B. Knight (LBP, NIAMDD); Dr. S. P. Nissley, Dr. A. C. Moses, Dr. R. M. White, and P. A. Short (Metabolism Branch, National Cancer Institute); Drs. C. R. Kahn and G. S. King (Diabetes Branch, NIAMDD)

Thyrotropin Receptors, Thyroid Regulation, and Pathophysiology

A model of the thyrotropin (TSH) receptor structure and the mechanism by which the receptor-TSH complex is coupled to the adenylate cyclase system has been proposed and evidence to support its outline obtained. In the course of this work, two new enzymes regulating thyroid cyclase activity have been defined; monoclonal antibodies to TSH receptors have been prepared and their relation to autoimmune antibodies defined; an endogenous thyroid lectin regulating TSH receptor expression has been discovered; a new solid phase TSH radioreceptor assay has been developed which is applicable to clinical use; and the action of synthetic peptides mimicking TSH cyclase action have been further defined.

. . . . . Drs. M. J. S. De Wolf, M. Epstein, G. Lee, F. S. Ambesi-Impiombato, Z. Yavin, E. Yavin, P. Vitti, D. L. Gill, and L. D. Kohn (LBP, NIAMDD); Dr. S. Shifrin (DCBD, NCI); Dr. M. D. Schneider (BG, IR, NHLBI); Dr. R. J. Winand (University of Leige, Belgium); Drs. S. M. Aloj, E. Consiglio, and M. F. Meldolesi, and F. Beguinot (University of Naples, Italy)

Tetanus Receptors and Mechanism of Action

Neural cells have been grown on defined media for the first time. Receptor-mediated tetanus toxin entrance into the cell and toxin association with cytoskeleton elements have been demonstrated.

. . . . . Drs. Z. Yavin, E. Yavin, and L. D. Kohn (LBP, NIAMDD); Dr. E. F. Grollman (LBP, NIAMDD); Drs. M. C. Hardegree and W. H. Habig (DBP, BB, FDA)

Interferon Receptors and Action

Interferon effects on thyroid cell function and TSH action as an antiviral agent support the idea of a relationship between cell regulation by glycoprotein hormones and antiviral protection by interferon.

. . . . . Drs. G. Lee, F. S. Ambesi-Impiombata, and L. D. Kohn (LBP, NIAMDD); Dr. R. M. Friedman (LEP, NIAMDD); Dr. S. M. Aloj (University of Naples, Italy)

Graves' Disease and Autoimmune Thyroiditis

Primary thyroid defects in thyroglobulin structure and processing have been defined in chickens with autoimmune thyroiditis and euthyroid Graves' disease as genetic traits. Antibodies inhibiting TSH binding and present in patients with primary hyperthyroidism have been shown to not necessarily be stimulators of thyroid function but blockers.

. . . . . Dr. S. Shifrin (DCBD, NCI); Drs. D. L. Gill, L. D. Kohn, and F. S. Ambesi-Impiombato (LBP, NIAMDD); Dr. R. S. Sundick (Wayne State University School of Medicine, Detroit, Michigan)

Thyroglobulin Biosynthesis and Degradation

Two separate membrane receptor interactions have been defined. One, involving N-acetylglucosamine residues on the thyroglobulin molecule, is correlated with the iodination-organification process. The second appears to involve a sugar-PO_4 residue on the thyroglobulin molecule; the relationship of this determinant to thyroglobulin structure or lysosomal processing is being questioned. Thyroglobulin "abnormalities" have been detected as primary defects in chicken models of autoimmune thyroid diseases and euthyroid Graves' disease (see above).

. . . . . Dr. S. Shifrin (DCBD, NCI); Drs. D. L. Gill, E. F. Grollman, E. Consiglio, and L. D. Kohn (LBP, NIAMDD); Dr. J. E. Rall (CEB, NIAMDD)

Hormonal Regulation of Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

Hormone, toxins, interferon, neurotropic agents, and other bioactive ligands interact with cell surfaces to initiate their action on cells. One means these ligands can transmit information is by altering electrochemical ion gradients, i.e., membrane potential and specific ion modulation. In current projects, cells from target tissues and plasma membrane vesicles from these cells are being used to study the effect of many diverse agents on membrane potential, calcium, and proton fluxes. Effects of thyrotropin, tetanus toxin, and interferon on these parameters have been delineated. Tetanus toxin induces dose-dependent changes in the membrane potential and calcium fluxes of brain synaptosomes, effects that are blocked by antitoxin and not seen with biologically inactivated toxin preparations. The mechanisms of calcium accumulation by brain synaptosomal membrane vesicles have been characterized in order to (i) more precisely define the site of tetanus toxin action and (ii) further define the role of the synaptosomal membrane in regulating intrasynaptosomal calcium levels important in neuronal cell function. Interferon and thyrotropin, as early events, alter the membrane potential of cells which

are biologically responsive. These effects are being related to the initiation of hormone, toxin, or interferon action at the level of the plasma membrane. Toxins are being studied since they are natural pathogenic agents which subvert normal hormonal regulatory mechanisms.

. . . . . Drs. E. F. Grollman and D. L. Gill

Bivalent Ligand Model for Hormone Action

A quantitative model for hormone action has been formulated, based upon the hypothesis that some types of hormones may be capable of simultaneously binding to two chemically and functionally distinct classes of sites on the cell surface. One class of sites, called receptor, binds hormone with high affinity and specificity. The other class of sites, called effector, is postulated to bind free hormone with low affinity and specificity, but to bind receptor-bound hormone with very high affinity. Steady-state response is assumed proportional to the number of effector sites binding hormone. The model is capable of qualitatively and semiquantitatively rationalizing a variety of reported observations on the binding and action of insulin and EGF in the presence and absence of crosslinking agents such as anti-hormone antibody, lectins, and anti-membrane-protein antibodies.

. . . . . Dr. A. P. Minton

VIII. BLOOD COAGULATION, TRANSGLUTAMINASE, AND RELATED FACTORS

Tissue Culture - Blood Coagulation and Related Factors

A possible intrinsic role of Factor XIII in utero-placental tissue has been implicated from the observation of repeated spontaneous abortion and severe decidual bleeding in patients which have congenital Factor XIII deficiencies. Human placenta has been shown to contain large quantities of this factor (enzyme). In studies on the mode of regulation of Factor XIII, utilizing rabbit as a model, the cellular origin of Factor XIII and other transglutaminases in rabbit uterine endometrial and myometrial cells in primary culture as well as whole uterus, were examined by immunofluorescence, enzyme activity, and partial chromatographic separation. In contrast to strong fluorescence obtained with rabbit megakaryocytes, immunofluorescence from endometrial and myometrial cells in various stages of culture appeared indifferent from other Factor XIII negative cells, such as fibroblasts, endothelial cells, etc. In sharp contrast to these results, Ca^{2+}-dependent, thrombin-stimulated, [^{3}H]-putrescine-incorporating activity was observed with both types of cells. Analysis of this transglutaminase activity by ion exchange chromatography, gel filtration, and immunoprecipitation showed that this enzyme activity was clearly identified with that of membrane-associated transglutaminase, not Factor XIII. These findings would suggest that the Factor XIII present in utero-placental tissues may not originate from the endometrial and/or myometrial cells, but may be supplied from other sources, for example, platelets.

Utilizing simian virus type 40 (SV-40), rabbit uterine endometrial and myometrial cells have been transformed at a temperature of 33° C. Eight to nine distinctive transformed cell lines have been obtained. One line, SV-2 RUE (for SV-40 transformed rabbit uterine epithelial cells), now possesses the capability of easy and rapid passage at 33° C or 37° C in a finite culture

media (modified Ham's F-12 with six hormones).

. . . . . Drs. J. A. Gladner, H. S. Sun, S. I. Chung (LB, NIDR), and H. G. Coon (LCB, NCI)

Modification of Fibrinogen-Thrombin Digestion of Citraconylated Canine Fibrinogen

The action of thrombin on citraconylated canine fibrinogen enzymatically released two fibrinopeptides. The rate of removal of modified fibrinopeptide-A was increased approximately 40% over the rate obtained for the action of thrombin on unmodified canine fibrinogen. The degree of citraconylation of canine fibrinogen regulated the rate of removal of fibrinopeptide-A, and at a modification level of 80 or more lysine groups modified inhibition of peptide release occurred. This inhibition could possibly be attributed to a conformational change in the structure of citraconylated fibrinogen brought about by the high incorporation of negative charge into the molecule. The site of thrombin action may have been masked and thereby reduced the efficiency of hydrolytic cleavage of the fibrinopeptide. Edman analysis verified the presence of a modified lysine in peptide-A, and additional confirmation was obtained from the inability of trypsin to hydrolyze the citraconylated fibrinopeptide-A.

Citraconylation of a Species of Fibrinogen Lacking Lysine in Fibrinopeptide-A

The citraconylation of human fibrinogen, a species lacking fibrinopeptide-A lysine, was accomplished for comparative analysis in order to implicate the additional negative charge imposed on canine fibrinopeptide-A, through modification, as the directing influence for the increased rate of release of the peptide from canine fibrinogen. The results demonstrated that the rate of release of fibrinopeptide-A by thrombin from citraconylated human fibrinogen, which had 40 lysine groups modified, was not significantly altered by modification. Therefore, although citraconylation of lysine residues had occurred elsewhere in the fibrinogen chains, it had neither accelerated nor inhibited the action of thrombin at the site of cleavage of the fibrinopeptide. This comparative study, therefore, confirmed our proposed mechanism that the increased activity of thrombin on citraconylated canine fibrinogen was a result of the incorporation of negative charge into the fibrinopeptide-A, at the lysine residue, resulting in a faster removal rate of the peptide by the hydrolytic cleavage of thrombin.

Structure-Function Relationship of Charge in the Fibrinopeptide Cleavage

Fibrinopeptide-A of canine and bovine fibrinogens is initially released at a faster rate than the B peptides by thrombin. The structure of each fibrinopeptide-A contained a carboxylic acid residue in the sixth position from the bond enzymatically cleaved by thrombin. Bovine fibrinopeptide-B is lacking a carboxylic acid residue at this location in the peptide; however, there is a lysine group in the seventh position which is citraconylated when the fibrinogen is modified. This procedure generated a negative charge at this position in the peptide which resulted in an increased rate of cleavage of the modified fibrinopeptide-B over and above the rate of release of unmodified peptide-B from unmodified bovine fibrinogen. Therefore, the presence of negative charge in the approximate vicinity of six residues removed from the bond hydrolyzed

by thrombin, influenced the enzymatic cleavage of an otherwise slower releasing fibrinopeptide from fibrinogen.

Removal of the Citraconyl Groups from Modified Fibrinogen

The citraconylation of fibrinogen was reversed in an effort to regenerate native fibrinogen. The addition of thrombin produced a fibrin clot and the release of two fibrinopeptides at the normal physiological rate. An electron microscopic study of the regenerated fibrin clot resulted in electron micrographs which were identical with unmodified fibrinogen-fibrin polymers. This reversibility was evidence that the citraconylation of lysine residues in the fibrinogen had not produced an irreversible conformational change which, upon regeneration of free ε-amino groups of lysine, would have interfered with the formation of normal periodicity during polymer formation.

Incorporation of Radioactive Glycine into Fibrinogen

Radioactive glycine ethyl ester was employed both to determine the free carboxyl groups remaining after methylation of fibrinogen, as well as, to label the carboxyl groups for structural identification of the carboxylic acid residues participating in the polymerization of methylated fibrinogen. The rate of fibrinopeptide-A released from methylated bovine fibrinogen was decreased 70% at approximately 100 residues modified in the fibrinogen. The composition of the methylated fibrinopeptide-A was determined to have four of its five carboxyl groups methylated. Structural analysis, after proteolytic cleavage of the peptide by chymotrypsin, identified the unmethylated carboxyl group which incorporated radioactive glycine as residing penultimate to the phenylalanine residue. On the other hand, the glutamic acid residue six positions removed from the enzymatically susceptible arginyl-glycyl bond, which was proteolytically cleaved by thrombin in the clotting of fibrinogen, was methylated. The modification of this glutamic acid residue was significantly implicated in the drastic reduction of thrombin activity toward fibrinogen.

. . . . . Dr. A. J. Osbahr III

Host Versus the Proliferating Cell

In the past several years we have been studying the role of transglutaminase in the proliferation of tumor cells in mice. Our experience is summarized with the proposition that cancer cells are able to cover-up their "strangeness" and "vulnerability" from T-lymphocytes with the use of transglutaminase (Factor XIII in blood plasma).

Here we report briefly that by inactivating transglutaminase with alkyl isocyanates in the tumor-bearing or pregnant mice, T-cells are generated which kill the unmasked tumor cells and the unmasked conceptus.

. . . . . Dr. K. Laki

The Cancer Cell. For these experiments we used CAF_1 mice and YPC-1 plasma cell tumors. The tumor-bearing mice were treated with BCNU or NCNU, one yielding chloroethyl isocyanate, the other neopentyl isocyanate. For the immunization, tumor cells (subcutaneous or intraperitoneal) were removed a few hours after BCNU or NCNU administration. These cells, inactivated either with radiation or mitomycin, were used in in vivo and subsequently in in vitro

sensitization of the splenic lymphocytes of normal mice. These sensitized lymphocytes specifically kill, in the 4-hour cytotoxic test, ^{51}Cr-labeled tumor cells: cold targets reduce ^{51}Cr release, while E14 cells do not.

Our finding that NCNU given to CDF_1 (Balb/c x DBA) and to CAF_1 (Balb/c x AL/n) eliminates implanted L1210, P388, YPC-1, and YLL tumors days after the decomposition of the drug, supports the view that it is the immune system which kills tumor cells devoid of their protective shield. The involvement of the immune system is further demonstrated by the finding that mice "cured" with these drugs will not take up the tumor.

. . . . . Drs. K. Laki, S. T. Yancey (LCHPH, NCI), T. Szabo, and G. Szegedi

The Conceptus. The role of transglutaminase in the proliferating conceptus is indicated by the finding that Factor XIII-deficient women not able to "mask" the fetus, abort it. (With the addition of Factor XIII, the pregnancy is successful.)

In cooperation with Drs. A. B. Mukherjee and A. K. Agrawal, we find that the conceptus taken out from the tuba of pregnant mice treated with NCNU 4 hours earlier, readily develops in tissue culture to the morula or blastocyst stage, while if left in the animal it would have been destroyed. This again indicates that it is not the drug which kills the conceptus, but the immune system. Recent experiments show that indeed conceptus "unmasked" by inhibiting transglutaminase, stimulates blast transformation of lymphocytes. Evidence can be marshalled to propose that in the case of the conceptus it is uteroglobin which is attached to the cell surface by transglutaminase (Factor XIII) in order to protect the fetus from the mother's immune system.

It appears that proliferating cells are "strangers" to the host organisms and that their strangeness (altered self) is masked by transglutaminase attaching a protein to the "modified self" antigen.

. . . . . Drs. K. Laki, A. B. Mukherjee (PRB, NICHHD), and A. K. Agrawal (PRB, NICHHD)

Beta-2-Microglobulin. Recognition and subsequent killing by T-lymphocytes involves the modified histocompability antigen (altered self) on the targets. In the proposition we are developing, the "self" modified by fetal antigens is a substrate of transglutaminase. One constitutent of the altered self is beta-2-microglobulin (β-2-m). We now find that β-2-m is a substrate of transglutaminase. By the action of the enzyme, large polymers of this small protein form; also, labeled methylamine becomes incorporated. Fluorescent antibody against β-2-m revealed that the even distribution of β-2-m on the cell surface becomes patchy after transglutaminase incubation.

. . . . . Drs. K. Laki, E. Fesus (LBC, NIAMDD), and L. Muszbek

IX. TRAUMATIC SHOCK AND CELLULAR IMMUNITY

Work on the defect in cellular immunity produced by extensive burns in mice has shown no significant impairment in the migration of peritoneal lymphocytes but a decreased migration of splenic lymphocytes. No decrease in lymphokine production was found. Studies on the effect of catecholamines and adrenergic blocking agents on shock mortality in mice, following burn and tourniquet

trauma and endotoxin, showed that epinephrine and norepinephrine increased mortality given as pretreatment and had no effect given after trauma. Propanolol decreased mortality as pretreatment but was ineffective later. Dibenamine lowered mortality from endotoxin shock. None of these agents prevented fall in body temperature.

Experiments are being continued on the relation of sodium chloride administered in the diet of normal mice on fluid movement, blood pressure, and longevity.

. . . . . Dr. S. M. Rosenthal

X. MYCOBACTERIUM

The present culture of growth of megakaryocytes shows two stages of growth: stimulative and degenerative. In the stimulative stage, the entire sequence of cellular events through to the formation and release of platelets were observed. In the degenerative stage, apparently all abnormal changes of megakaryocytes reported in various thrombocytopoietic disorders were also observed in the cultures. Thus, this culture may be used as an in vitro model for studies of thrombocytopoietic disorders, especially for the idiopathic thrombocytopenic purpura, which shows both stimulative and degenerative changes in megakaryocytes.

. . . . . Dr. Y. T. Chang

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 23,140-22 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemistry of Sulfur-Containing Compounds

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Simon Black, Ph.D.	Biochemist	LBP NIAMDD
	and Chief, Section on Biochemistry of Amino Acids		LBP NIAMDD
OTHER:	Chien-Hua Niu, Ph.D.	Staff Fellow	LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

I. Aminoacyl tRNA synthetase regulation. A heat-stabile, nondialyzable acidic substance has been found which stimulates a valyl tRNA synthetase from yeast.

II. Evolution. Hydrogen bonds between the cytosine ring and peptides have been identified. Three such bonds form with each cytosine molecule, and are particularly strong when the peptide contains serine or threonine.

PHS-6040

Project Description:

I. Protein Synthesis. The aminoacyl tRNA synthetases of all living organisms catalyze the following general reaction:

amino acid + ATP + tRNA $\rightleftharpoons$ aminoacyl tRNA + AMP + PP.

We have found previously that within the yeast cell two forms of the glycyl tRNA synthetase co-exist, one being dependent on activator-like substances, whereas the other is active in the absence of any special additives. These two forms appear to be cyclically interconverted during growth as part of a regulatory mechanism. More recently the activity of a valyl tRNA synthetase of yeast was found to be enhanced by a heat-stable, nondialyzable acidic substance obtained from crude extracts. The purification and characterization of this substance is now our major objective.

We plan to continue this study with the general objective of elucidating the detailed components and operation of the mechanism of this regulatory phenomenon in the first step of protein synthesis.

II. Evolution. Experiments designed to elucidate the origin of the genetic code have been continued. Demonstrations of hydrogen bond formation between uracil and peptides were made previously. New experiments have shown a very strong binding of cytosine derivatives to peptides, particularly to peptides of serine and threonine. These results have an important bearing on the origin of codons with cytosine as the central base.

Publications:

Black, S.: Molecules in space and life on earth. Trends Biochem. Sci. 4: N271, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,230-30 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chemotherapy of Mouse Leprosy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Yao Teh Chang, M.D. Research Pharmacologist LBP NIAMDD

COOPERATING UNITS (if any)

Dr. Ephraim Yavin, Section on Biochemistry of Cell Regulation, LBP

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Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The present culture of growth of megakaryocytes shows two stages of growth: stimulative and degenerative. In the stimulative stage, the entire sequence of cellular events through to the formation and release of platelets were observed. In the degenerative stage, apparently all abnormal changes of megakaryocytes reported in various thrombocytopoietic disorders were also observed in the cultures. Thus, this culture may be used as an in vitro model for studies of thrombocytopoietic disorders, especially for the idiopathic thrombocytopenic purpura, which shows both stimulative and degenerative changes in megakaryocytes.

Project Description:

Growth of Megakaryocytes in Cultures

Studies on the growth of megakaryocytes in cultures was completed. The number of megakaryocytes increased 4- to 8-fold in a period of 4 days. A linear relationship was observed between the number of megakaryocytes produced and the number of marrow cells seeded. A full cell cycle of growth of megakaryocytes was observed. The entire sequence of cellular development was as follows: megakaryoblasts, promegakaryocytes, endomitosis, granulopoiesis, formation of demarcation membrane system, and platelet liberation. The cell cycle lasted for 5-6 days.

Apart from the full cell cycle, two incomplete cell cycles were also observed: the final stages of cell development on the first day and the early stages of cell development at 9 to 13 days of cultivation.

There were two types of growth of megakaryocytes in the cultures: stimulative and degenerative. During the stimulative growth many macromegakaryoblasts and macropromegakaryocytes appeared in the cultures. The number of these cells decreased progressively, but a few cells were still observed as late as 13 days after cultivation. During the degenerative stage, cells revealed various destructive processes: i.e., defect in granulopoiesis; dissociation of nucleus-cytoplasm development; appearance of micromegakaryocytes; vacuolation, degranulation, hyalinization, and fragmentation of the cytoplasm; and pyknosis, karyorrexis, and diffused degeneration of the nucleus. It should be noted that all these findings have been observed in various clinical thrombocytopoietic disorders, especially in the idiopathic thrombocytopenic purpura, in which both stimulation and degeneration in the growth of megakaryocytes were observed in the patients.

Current studies on the growth of megakaryocytes require both the semi-solid medium (i.e., soft-agar, methylcellulose, or plasma clot), and the growth-stimulating factors (i.e., erythropoietin, conditioned media from cultures of various tissues or cell lines) in the cultures. Cultures contain various sizes of cell colonies which often mixed up with other types of cells. This makes cell enumeration rather difficult. Furthermore, a full cell cycle of growth of megakaryocytes has not yet succeeded, although some cell fragments (believed to be the released platelets) have been observed with electron microscopy.

In the present cultures, cells were grown in a liquid medium. Megakaryocytes were stained with MGG on well-spread cell preparations made with cytocentrifuge. Details of cytoplasmic morphology were well exhibited. Evaluation on the growth of cells was not influenced by the presence of growth-stimulating factors in the medium nor by the differences in the size and cell types of the cell colonies. Quantitative evaluation on the growth of cells could be made at each step of cellular development. Agents which could improve the growth at any stage of the cell cycle, e.g., granulopoiesis or demarcation

membrane synthesis, would be of great value for clinical investigations.

Cinemicrographic Studies on the Growth of Neurons

Cinemicrographic studies on the growth of cerebral neurons in cultures are in progress. This is a cooperative study with Dr. Ephraim Yavin (Section on Biochemistry of Cell Regulation, LBP).

Studies on a Chinese Anti-Inflammation Drug, Lei-Kung-Teng

Negotiation for a cooperative study with investigators in the People's Republic of China is in progress. This will be a study of a herb drug which is used in China for treatment of the notorious lepra reaction in leprosy patients. This herb drug is claimed to be better than cortisone and at least as good as Thalidomide, the standard drug currently in use for the treatment of lepra reaction.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 23,330-02 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Aldoheptose Biosynthesis and Its Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William G. Coleman, Jr., Ph.D.	Research Microbiologist	LBP NIAMDD
OTHER:	Ellis L. Kline, Ph.D.	Intergovernmental Personnel Act Appointee	LBP NIAMDD

COOPERATING UNITS (if any)

None

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.5	1.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A mutant of Escherichia coli K-12 carrying a mutation, designated rfaD, which affects the synthesis of the aldoheptose, L-glycero-D-mannoheptose, has been isolated. It was observed that the lipopolysaccharide of the rfaD mutant contained the aldoheptose D-glycero-D-mannoheptose rather than the normal L-glycero-D-mannoheptose. It is postulated that L-glycero-D-mannoheptose is formed from D-glycero-D-mannoheptose via a racemization. A crude assay to measure the epimerase activity has been developed. Hybrid colicin E1 plasmids capable of correcting the rfaD phenotype have been selected, and these increase the epimerase activity 6- to 10-fold relative to the wild type. This represents the initial step in understanding the biochemistry of aldoheptose biosynthesis and provides a system for studying its regulation. We previously selected from the Clark and Carbon colony bank, hybrid ColE1 plasmids which corrected the rfaD phenotype and conferred ColE1 resistance by F-factor--mediated transfer. This was further demonstrated by transformation experiments with purified plasmids as DNA sources. We are now characterizing the hybrid ColE1 plasmids and cloning and fine mapping the rfaD gene.

PHS-6040

Project Description:

Objectives:

(1) To elucidate and characterize the biochemical steps leading from sedoheptulose-7-P to L-glycero-D-mannoheptose. (2) To study the regulatory mechanisms involved in these steps. (3) To isolate and clone genes necessary for this process. (4) To measure, isolate, and purify the proteins involved in aldoheptose biosynthesis.

Major Findings:

(1) A mutant of E. coli K-12 altered in the final step of aldoheptose biosynthesis has been isolated and genetically and biochemically characterized.

(2) Temperature-sensitive mutants with genetic alterations which affect aldoheptose biosynthesis have been isolated.

(3) Hybrid ColE1 plasmids capable of complementing the aldoheptose deficiency have been selected. In addition, strains carrying these plasmids produce 6- to 10-fold more enzyme than the parent strains. These plasmids, when purified, can be used to transform aldoheptose mutants to wild type.

Publications:

Coleman, W. G., Jr., and Leive, L.: Two mutations which affect the barrier function of the Escherichia coli K-12 outer membrane. J. Bacteriol. 139: 899-910, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 23,580-17 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Protein Synthesis and Gene Expression in Escherichia coli and Other Organisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Anthony V. Furano, M.D.	Medical Officer (Research)	LBP NIAMDD
OTHERS:	David Filer, Ph.D.	Visiting Fellow	LBP NIAMDD
	Fredric S. Young, Ph.D.	Staff Fellow	LBP NIAMDD
	Frank R. Witney, Ph.D.	Staff Fellow	LBP NIAMDD

COOPERATING UNITS (if any)
Dr. Lloyd Greene, Department of Pharmacology, New York University Medical School, New York, New York

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.2	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are continuing our investigations into the regulation of protein synthesis and gene expression in Escherichia coli. In addition, we have extended our studies to examine the mechanism whereby nerve growth factor (NGF) induces the differentiation of immature neural cells into mature neurons. The studies on NGF are being carried out with the collaboration of Dr. Lloyd Greene.

PHS-6040

Project Description:

I. Evolutionary Studies

A wide variety of completely unrelated prokaryotes share a set of DNA sequences that are highly homologous to the Escherichia coli tuf gene. In E. coli, this gene encodes peptide chain elongation factor Tu that binds aminoacyl tRNA to the A-site of the ribosome.

This type of sequence conservation is unique to the tuf gene, a finding that suggests that the widespread presence of this set of highly conserved tuf-homologous sequences is not due to recent gene transfer among prokaryotes but is due to strong selective pressure. Due to the degeneracy of the genetic code, DNA sequences need not and have not been found to be conserved in order to conserve protein sequence. Therefore, the conservation of tuf-homologous sequences could mean that evolutionary selection can operate at the level of codon utilization.

We have found duplicate tuf genes in every gram-negative organism examined (10 enteric, 5 nonenteric) but only one tuf gene in the two gram-positive organisms that we tested. By correlating the number and chromosomal proximity of the tuf genes with the linkage maps and phylogenetic relationship of the organisms that we have examined, we postulate the following scenario for the evolution of the tuf gene duplication. Approximately one billion years ago when the gram-positive and gram-negative branches diverged, the tuf gene was duplicated in one of the early gram-negative progenitors. The duplicated tuf genes remained in close proximity on the chromosome until the time when the more recently evolved genera arose, such as the enteric family, when one of the tuf genes and a portion of the nearby chromosome was translocated to a distant part of the chromosome.

II. Studies on tuf Gene Regulation

The synthesis of EF-Tu is regulated in part at the level of translation. tuf mRNA (especially that produced from the tufA gene) is translated at least 3 times more efficiently than any other mRNA, including the one for EF-G, and our finding completely explains how the cell can maintain the 8- to 14-fold molar ratio of EF-Tu to the other proteins required for peptide chain elongation.

Part of the EF-Tu mRNA (which is 1,200 bases long) is produced from the tufA gene by a specific endonucleolytic cleavage of the large (4,000 bases long) transcript of the operon which contains the tufA gene. This is the first example of post-transcriptional modification of mRNA molecules in prokaryotes and may be related to the high translational efficiency of the tufA gene transcript.

The transcriptional activity of the operon that contains the tufA gene (also called the str operon) in some way regulates the activity of all of the rRNA

gene operons. We find that cells which cannot decrease transscription of the tufA-operon cannot decrease the synthesis of rRNA even under conditions of cell growth that in normal cells markedly decrease the synthesis of rRNA.

We have recently determined the DNA sequence of the beginning of the tufA gene with the aim of trying to understand the structural basis for the high translational efficiency of the tufA mRNA. Unexpectedly, we found that some of the DNA sequence does not correspond to that which would encode the amino acid sequence of EF-Tu. These findings imply that part of the sequence for this part of the EF-Tu molecule may be derived from elsewhere in the genome. We are currently determining the DNA sequence of the corresponding part of the tufB gene.

III. Mechanism of Action of Nerve Growth Factor and the Regulation of Eukaryotic Genes Involved in Cell Development

We have recently begun a program to identify and isolate those rat genes that must be expressed to attain or maintain a particular state of cellular development or growth. We are using a cloned pheochromocytoma cell line, pC12, that can respond to nerve growth factor by differentiation from a dividing cell to a nongrowing, electrically excitable neuronal-like cell, as well as regenerating rat liver cells as model systems. We are now in the process of generating three genomic libraries from rat DNA: one comprising the entire genome, one comprising just the repetitive DNA fraction, and one consisting of the repetitive DNA and the single copy DNA that is contiguous with the repetive DNA. So far we have found that there are differences between the small nuclear RNA population of nondividing and dividing hepatocytes as well as between these RNA's and those found in the pC12 line. We have also found that significantly different and nonoverlapping regions of the rat genome are expressed in rat liver cell and the pC12 cells.

Publications:

Filer, D., and Furano, A. V.: Portions of the gene encoding elongation factor Tu are highly conserved in prokaryotes. J. Biol. Chem. 255: 728-734, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,630-15 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biology of Complex Carbohydrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor Ginsburg, Ph.D.	Research Chemist	LBP NIAMDD
	and Chief, Section on Biochemistry		LBP NIAMDD
OTHERS:	David F. Smith, Ph.D.	Senior Staff Fellow	LBP NIAMDD
	John L. Magnani, Ph.D.	Staff Fellow	LBP NIAMDD
	Concepcion V. Warner, Ph.D.	Staff Fellow	LBP NIAMDD
	Manfred B. Brockhaus, M.D.	Guest Worker	LBP NIAMDD

COOPERATING UNITS (if any)
None

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Section on Biochemistry

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.7	3.7	2.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oligosaccharides will be isolated from natural sources. Oligosaccharides will be coupled to macromolecules in order to make antibodies specific for certain sugar sequences. The antibodies will be used in radioimmune assays for the occurrence of these sugar sequences in the glycolipids and glycoproteins of cell membranes and serum in order to investigate the involvement of the complex carbohydrates of cell surfaces in development and regulation or as tumor-specific antigens.

Waldenström macroglobulins and myeloma proteins will be screened for their ability to react with human erythrocytes. Those that do will be tested for their ability to react with various oligosaccharides coupled to macromolecules.

Carbohydrates on cell membranes that are receptors for toxins, hormones, and antibodies will be isolated and characterized.

Project Description;

Many carbohydrates of cell membranes occur at levels too low to be detected by conventional means. We are developing immunological methods for their detection using two general approaches for obtaining carbohydrate-specific antibodies: (1) animals will be immunized with pure oligosaccharides coupled to macromolecules and (2) the specificities of Waldenström macroglobulins and myeloma proteins which bind carbohydrates will be determined.

Gangliosides that bind cholera toxin can be detected by the direct binding of ^{125}I-labeled toxin to thin-layer chromatograms followed by autoradiography. The method is very sensitive: 0.1 ng of G_{M1} (70 femtomoles) can be detected on chromatograms of total lipid extracts of cells. The method may be useful in defining the receptors of other toxins or proteins such as some lectins, antibodies, and hormones that bind carbohydrates.

Anti-lacto-N-difucohexaose I IgG purified by affinity chromatography from the serum of a goat immunized with lacto-N-difucohexaose I coupled to polylysine is specific for the Le^b antigen, but its usefulness for the routine determination of Lewis blood type is limited because the antibody does not agglutinate human erythrocytes. However, the antibody can be converted into a powerful Le^b agglutinin by polymerization with dimethyl suberimidate.

In an attempt to provide an explanation for the previously-reported effect of pregnancy on the Lewis phenotype of erythrocytes, the level of Le^b-active glycolipid in serum was compared with the reactions of erythrocytes, using samples obtained from 73 non-pregnant women, 74 women at the time of delivery, and 2 women at weekly intervals during their pregnancy. In this Swedish population, the frequency of Le(a-b-) blood type increased from 11% in non-pregnant women to 36% in women at the time of delivery. Among Le(a-b+) women of all ABO types, those who were A_1 most often became Le(b-) during pregnancy. The change in phenotype occurred as early as the 24th week of gestation; the Le^b antigen was again detectable within six weeks after delivery. The concentration of Le^b glycolipid in serum, as measured by radioimmunoassay, decreased only slightly during pregnancy. A repartition of glycolipids, secondary to the increased ratio of lipoprotein to red cell mass which occurs during pregnancy, may account for the relative lack of Lewis glycolipid on erythrocytes.

Proposed Course:

Oligosaccharides will be isolated from natural sources. Certain oligosaccharides will be coupled to macromolecules in order to make antibodies specific for certain sugar sequences. The antibodies will be used in radioimmune assays for the occurrence of these sugar sequences in the glycolipids and glycoproteins of cell membranes and serum.

Waldenström macroglobulins and myeloma proteins will be screened for their ability to react with human erythrocytes. Those that do will be tested for

their ability to react with various oligosaccharides coupled to macromolecules in order to define their specificity.

Carbohydrates on cell membranes that are receptors for toxins, hormones, and antibodies will be isolated and characterized.

Publications:

Ginsburg, V., McGinniss, M. M., and Zopf, D. A.: Biochemical basis for some blood groups. In Sandler, S. G., Nusbacher, J., and Schanfield, M. S. (Eds.): Immunobiology of the Erythrocyte, Progress in Clinical and Biological Research Series. New York, Alan R. Liss, Inc., 1980, in press.

Rohr, T. E., Smith, D. F., Zopf, D. A., and Ginsburg, V.: Le^b-Active glycolipid in human plasma: measurement by radioimmunoassay. Arch. Biochem. Biophys. 199: 265-269, 1980.

Smith, D. F., and Ginsburg, V.: Antibodies against sialyloligosaccharides coupled to protein. J. Biol. Chem. 255: 55-59, 1980.

Warner, C. V., Smith, D. F., Zopf, D. A., and Ginsburg, V.: A human Le(a-b+) blood group agglutinin prepared by polymerizing goat anti-lacto-N-difucohexaose I IgG. Mol. Immunol., in press.

Hammer, L., Månsson, S., Rohr, T., Chester, M. A., Ginsburg, V., Lundblad, A., and Zopf, D.: Lewis phenotype of erythrocytes and Le^b-active glycolipid in serum of pregnant women. Vox Sang., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 23,730-20 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Factors Involved in Blood Coagulation and Related Fields

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	3.3	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tissue culture of rabbit uterine endometrial and myometrial cells have been utilized to investigate the cellular origin of utero-placental coagulation factor XIII. An SV-40 transformation of these endometrial cells has been successful. Laser light scattering is being utilized to study rigidity of noncrosslinked and crosslinked clots of lamprey fibrin as well as the plasmin degradation of crosslinked bovine fibrin clots. Salmon plasmin of very high activity is being isolated and characterized.

Project Description:

Objectives:

The final phases of protein-protein interactions in the "cascade scheme" of blood coagulation lead to fibrin formation via thrombin action. Fibrin clots are then stabilized by isopeptide bonds (crosslinking) formed between fibrin monomers mediated by coagulation factor XIII. These biomolecular reactions have been examined in lower vertebrates in order to elucidate possible differences and/or similarities in molecular structure and action which may be useful in understanding and treating disorders in man. Additionally, factor XIII and like transglutaminases may play a major role in other processes where protein stabilization through crosslinking are implicated such as wound healing, conception, etc. Investigations have now been initiated as to the biosynthesis, regulation, storage, and release of factor XIII in tissue cultures, i.e., dynamic systems.

Methods:

All applicable methodology of protein and enzyme isolation, purification, and characterization are employed. Physical biochemistry includes ultracentrifugation and laser light scattering. In conjunction with tissue culture studies, radioactive assays include incorporation and radioactive immunoassays. Fluorescent antibody (FITC) techniques are also utilized.

Resumé of Ongoing Investigations:

I. Tissue Culture

Coagulation factor XIII (protransglutaminase) in vertebrates are mostly present in plasma and platelets. Human placenta and uterus also contain significant amounts of factor XIII. A possible intrinsic role of the enzyme in utero-placental tissue has been implicated from the observation of repeated spontaneous abortion and severe decidual bleeding in congenital factor XIII-deficient patients. Factor XIII isolated from platelets consists of dimeric catalytic subunits, whereas plasma factor XIII possesses additional dimeric carrier subunits. Factor XIII isolated from human uterus and placenta were characterized to be composed of mostly catalytic subunits with properties similar to that of the intracellular platelet zymogen. In continuing studies on the possible mode of regulation of factor XIII (utilizing rabbit as a model), the cellular origin of factor XIII and other transglutaminases in uterine endometrial and stromal cells in culture as well as whole uterus were examined by enzyme activity, immunofluorescence, and partial chromatographic separation.

Cells were isolated from inverted rabbit uteri in a dissociation media consisting of 0.075% trypsin, 0.05% collagenase, 0.2% chick serum v/v in HBSS, at 37°. Cells were harvested in short time sequences. The majority of the cells from the first two harvests were primarily epithelial cells. In subsequent

harvests, stromal cells predominate. Homogeneous stromal cells were obtained following cloning. Both cell types were cultured in modified Ham's F-12 media with 0.5% calf serum v/v, antibiotics and insulin, thyrotropin, hydrocortisone, transferrin, somatostatin, and GHL. These epithelial cells could be maintained in cultural conditions for 3 months but were difficult to pass. All experiments were performed on primary cultures. Transglutaminase activities were assayed by [^{3}H]putrescine incorporation into casein in the presence of Ca^{2+} and thrombin. Immunofluorescence staining employed monospecific FAB anti-rabbit factor XIII from goat used as primary antibody. FITC-anti-goat IgG (FAB) from rabbit was utilized as the second antibody. Goat anti-rabbit factor XIII IgG was treated with cell and tissue extracts, and transglutaminase was assayed in the supernatant (antibody inhibition of transglutaminase).

Endometrial (epithelial) and stromal cells in various stages of culture appeared indifferent from other factor XIII-negative cells. However, Ca^{2+}-dependent, thrombin-stimulated [^{3}H]putrescine incorporation activity was observed with both cell types. Subsequent analysis of this transglutaminase by ion exchange chromatography, gel filtration, and immunoprecipitation showed that this enzyme activity was clearly identified with that of membrane-associated transglutaminase, not factor XIII. These findings suggest that the factor XIII present in utero-placental tissues does not originate from the endometrial and myometrial cells, but is supplied from other sources, i.e., platelets.

Simian virus type 40 (SV-40) has been used to transform rabbit uterine endometrial and myometrial cells at a temperature of 33°. Eight to nine distinctive cell lines have been cloned. One line, named "SV-2 RUE," resembles the primary epithelial cells. It possesses the capability of easy and rapid passage at 33° and 37° in the modified Ham's F-12 culture media described above. Preliminary experiments with fluorescent antifibronectin antibody indicate the presence of fibronectin in cell cultures of these transformed cells. The possible source of this protein is being carefully examined.

II. Laser Light Scattering

Lamprey fibrinogen and fibrin appear to differ in structure and mode of crosslinking from higher species. Initial studies to determine the shear modulus of noncrosslinked and crosslinked lamprey fibrin is in progress. The degree of plasmin degradation of crosslinked bovine and human fibrin clots as measured by change in shear modulus is also under study by this method.

III. Salmon Plasminogen and Plasmin

Further purification of highly active salmon plasmin is proceeding slowly due to the variance of different standard assay systems. In an effort to compare salmon plasmin with plasmins of other species (human and bovine), ^{125}I-labeled and ^{3}H-labeled fibrin plates are being examined as more definitive substrates.

IV. Lamprey Fibrinogen

Characterization of the Aα entity of this protein is the object of concentrated investigation. It has not been possible to identify the number of peptides postulated to be obtainable by complete cyanogen bromide hydrolysis. This could be based on a proposed structure containing isopeptide bonds, but evidence is lacking.

Significance to Biomedical Research and the Program of the Institute:

Characterization of blood coagulation factors and their mechanism of action are critical to our knowledge of cardiovascular abnormalities which may result in infarctions and strokes. Protein stabilization via crosslinking reactions by factor XIII and similar type enzymes appear to be involved in these and other physiological processes such as wound healing, conception, etc. Studying biosynthesis and regulation of factor XIII in organ tissue culture, uterus, will give greater knowledge of this factor in a physiological medium.

Proposed Course:

Coagulation factor XIII, and others, from cellular culture will be stressed in addition to the projects described above.

Publication:

Gelman, R. A., Gladner, J. A., and Nossal, R. J.: The rigidity of fibrin gels as measured by quasielastic light scattering. Biopolymers 19: 1259-1270, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 23,830-01 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.5	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hormone, toxins, interferon, neurotropic agents, and other bioactive ligands interact with cell surfaces to initiate their action on cells. One means these ligands can transmit information is by altering electrochemical ion gradients. In current projects, cells from target tissues and plasma membrane vesicles from these cells are being used to study effects on membrane potential, calcium, and proton fluxes. Effects of thyrotropin, tetanus toxin, and interferon on these parameters have been delineated. Tetanus toxin induces dose-dependent changes in the membrane potential and calcium fluxes of brain synaptosomes. The mechanisms of calcium accumulation by brain synaptosomal membrane vesicles have been characterized in order to define the site of tetanus toxin action and the role of the membrane in regulating intrasynaptosomal calcium. Interferon and thyrotropin, as early events, alter the membrane potential of cells which are biologically responsive. These effects are being related to the initiation of hormone, toxin, or interferon action at the plasma membrane level. Toxins are being studied as they are natural pathogenic agents which subvert normal hormonal regulatory mechanisms.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

From work in this section (Section on Biochemistry of Cell Regulation) the idea has developed that the receptors for thyrotropin (TSH), toxins (tetanus and cholera), and the antiviral agent interferon are structurally and functionally related. A pathophysiological action that these agents have in common is an effect on ion gradients. My work has been to further define these effects, i.e., the effects of TSH, interferon, and tetanus toxin on membrane potential and ion fluxes. The work has also evolved in related areas: (i) study of the effects on other hormones such as insulin and glucagon and pharmacological agents on ion transport and (ii) the role of electrochemical ion gradients in active transport and metabolism of diverse mammalian cell types.

Methods Employed:

The methods used in bacterial cell transport have been adapted to studies of transport in mammalian cells isolated from target tissues or grown in culture. Plasma membrane vesicles, devoid of internal organelles, have been prepared from these cells, as this allows manipulation of transmembrane ion gradients and focuses on membrane-induced events. Radioactive, fluorometric, and colorometric indicators have been used to determine membrane potential, internal pH, and proton and cation fluxes.

Major Findings:

(1) TSH alters the electrical potential in cultured thyroid cells and in plasma membrane vesicles prepared from these cells. This effect of TSH is not mediated by cAMP and precedes it in time. Cloned thyroid cells which differ in their iodide uptake and utilization are being characterized in their transport features, and these features measured are being related to iodide uptake processes.

(2) A synaptosome preparation has been developed that responds to tetanus and neurotoxins with alterations in electrochemical ion gradients. Tetanus toxin has been shown to hyperpolarize this preparation and also to cause a dose-dependent efflux of calcium. These effects on membrane potential and calcium movement are (a) abolished by tetanus antitoxin and (b) do not depend on functioning mitochondria.

(3) Calcium transport mechanisms have been described using membrane vesicles prepared from synaptosomes. Two major mechanisms of calcium transport exist: sodium-calcium exchange and ATP-dependent calcium uptake. Although independent, both mechanisms are coordinately regulated. When exposed to tetanus toxin, an effect on sodium-calcium exchange in these vesicles is seen but not on ATP-dependent calcium exchange. These experiments have defined for the first time an *in vitro* biochemical effect of tetanus toxin. The relationships

between tetanus toxin and TSH suggest that these approaches will be meaningful to the mechanism of thyroid hormone control.

(4) Interferon hyperpolarizes interferon-sensitive cells and vesicles. This change in electrical potential is associated with: (a) an effect on proton release from the cell with an increase in internal pH; (b) release of calcium from the membrane without an effect on calcium uptake; and (c) effect on amino acid uptake by cells and vesicles. These effects are not seen in interferon-insensitive cells which bind interferon, and these effects are abolished by antibodies to interferon.

Proposed Course:

The effect of tetanus toxin and other neurotoxins on calcium transport will be further characterized. The hormone-interferon effects on transport in mammalian cells will be continued and will be expanded to include other hormonal, toxic, and pharmacological agents. The membrane enzymes responsible for ion gradients will be measured and correlated with the hormone-toxin effects already observed. The relationship of the receptor and alterations of the receptor to ion transport will also be pursued.

Publications:

Aloj, S. M., Lee, G., Grollman, E. F., Beguinot, F., Consiglio, E., and Kohn, L. D.: Role of phospholipids in the structure and function of the thyrotropin receptor. J. Biol. Chem. 254: 9040-9049, 1979.

Gill, D. L., and Grollman, E. F.: Calcium transport mechanisms in membrane vesicles from guinea pig. J. Biol. Chem. 255, 1980, in press.

Grollman, E. F., Gill, D. L., and Dyer, S. A.: Effect of tetanus toxin on the accumulation of calcium by synaptosomes of mouse brain. Arch. Biochem. Biophys., in press.

Habig, W. H., Hardegree, M. C., Grollman, E. F., Lee, G., Ramos, S., and Kohn, L. D.: Interaction of tetanus toxin with thyroid membranes. Proceedings of the 5th International Tetanus Conference, Stockholm, Sweden, June 18-23, 1978, in press.

Kohn, L. D., Consiglio, E., De Wolf, M. J. S., Grollman, E. F., Ledley, F., Lee, G., and Morris, N. P.: Thyrotropin receptors and gangliosides. In Dreyfus, H., Mandel, P., Svennerholm, L., and Urban, D. F. (Eds.): Proceedings of the International CNRS Symposium on Structure and Function of Gangliosides, Bischenberg (Strasbourg), France, April 23-27, 1979. New York, Plenum Press, in press.

Lee, G., Grollman, E. F., Dyer, S., Beguinot, F., Kohn, L. D., Habig, W. H., and Hardegree, M. C.: Tetanus toxin and thyrotropin interactions with rat membrane preparations. J. Biol. Chem. 254: 3826-3832, 1979.

Z01 AM 23,830-01 LBP

Ramos, S., Grollman, E. F., Lazo, P. S., Dyer, S. A., Habig, W. H., Hardegree, M. C., Kaback, H. R., and Kohn, L. D.: Effect of tetanus toxin on the accumulation of a permeant lipophilic cation tetraphenylphosphonium by guinea pig brain synaptosomes. Proc. Natl. Acad. Sci. U.S.A. 76: 4783-4787, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 23,960-14 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cell Regulation by the Action of Pharmacodynamic Agents on the Cell Membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Eduardo Consiglio, M.D.	Visiting Scientist	LBP NIAMDD
	Ephraim Yavin, Ph.D.	Visiting Scientist	LBP NIAMDD
	Ziva Yavin, Ph.D.	Visiting Associate	LBP NIAMDD
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	Michael Epstein, Ph.D.	Intergovt.Personnel Act Appointee	LBP NIAMDD
	William A. Valente, M.D.	"	LBP NIAMDD
	Marc J. S. De Wolf, Ph.D.	Guest Worker	LBP NIAMDD
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9.6	8.5	1.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structural and functional relationship in the mechanism by which glycoprotein hormones [thyrotropin (TSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), and follicle-stimulating hormone (FSH)], certain bacterial toxins (cholera and tetanus, for example), and the antiviral protective agent, interferon, interact with and transmit their message through the cell membrane has been further defined. Studies have continued which explore the importance of these relationships to the expression of thyroid hyperfunction in Graves' disease; to organ-specific autoimmunity in general and the autoimmunity of Graves' disease in particular; to thyroid storm and the sympathetic overactivity syndrome of tetanus; to the ability of hormones to modulate the oncogenic state; and to the mechanism by which toxins subvert normal mechanisms to impose their pathological effects. Studies have been continued which evaluate the role of membranes in thyroglobulin biosynthesis and thyroglobulin biodegradation to T_3 and T_4 and the role of carbohydrate moieties in thyroglobulin structure and post-translational processing. Finally, studies have been initiated which concern lipid regulation of receptor expression with special emphasis on neuronal and thyroid cell development.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The thrust of work in this section is toward an understanding of the mechanisms by which hormonal and pharmacological agents regulate cell activity and the means by which these mechanisms are subverted by pathologic agents to express themselves in metabolic diseases. The specific theme of this element of the section centers around the understanding of thyroid physiology, development, and regulation with respect to normal body function and those pathologic states which subvert normal mechanisms. A basic biochemical and basic molecular biology approach is combined with attempts to express results in immediately relevant diagnostic or therapeutic systems which can redress normal growth and development.

Current efforts center upon the action of hormonal and pharmacological agents on cell membranes and the means by which these agents transmit information across membranes to effect normal cell functions or to subvert these functions. Since these efforts have defined a relationship in the structure and function of surface membrane receptors and transmission processes among thyrotropin, nonapeptide hormones, tetanus toxin, cholera toxin, nerve growth factor, interferon, and several plant lectins which affect mitogenic activity, as well as other glycoprotein hormones, work has extended to studies of these agents, their target tissues, their mechanism of action, and their interrelationships. In this regard, relationships between digestive diseases (cholera), thyroid metabolic diseases (primarily hypothyroidism, hyperthyroidism, thyroid storm, Hashimoto's disease), and neuronal diseases (tetanus) are being explored.

Current efforts toward diagnostic and therapeutic applications center on the synthesis and development of small peptides which can effect hormonal functions; the development of monoclonal antibodies to measure thyroid-stimulating or thyroid-blocking antibodies in primary hyperthyroidism; receptor function in primary hyperthyroidism or hypothyroidism; and the evaluation of membrane factors which can enhance the antiviral protective effect of interferon 10- to 100-fold.

Major Findings:

I. Definition of the Structure and Function of the Thyrotropin (TSH) Receptor.

The TSH receptor appears to be composed of both a glycoprotein and a ganglioside. Evidence for the existence of a glycoprotein component includes the ability of trypsin to release a fragment of this component from target cells with attendant losses in adenylate cyclase stimulation or alterations in electrochemical ion gradients. Evidence also includes isolation after solubilization and reconstitution of binding functions in liposomes. Evidence for the role of gangliosides includes the demonstration of ligand-ganglioside interactions; specificity in these interactions; the presence of gangliosides in biologically active systems but their absence in biologically inactive

systems which can even have a normal glycoprotein component. The glycoprotein is believed to be a high affinity primary binding component which is able to distinguish between TSH and nonrelated polypeptide hormones. The ganglioside is believed to carry out the following functions: (1) Complete specificity by distinguishing among glycoprotein hormones and related ligands such as tetanus toxin, cholera toxin, and interferon. (2) Modulate the apparent affinity and capacity of the glycoprotein receptor component. (3) Induce a conformational change in the hormone believed necessary for subsequent message transmission. (4) Form an anhydrous complex between the hormone and the lipid bilayer of the membrane. (5) Allow the ligand to perturb the phospholipid bilayer through alterations in lipid order. (6) Alter the ion flux across the membrane. In a sense, therefore, the model proposes that the glycoprotein receptor component is high affinity "fly paper" attaching the hormone to the cell surface, whereas the ganglioside acts as an emulsifying agent which allows the hormone to interact with other membrane components in the lipid bilayer.

In this year the following studies have been accomplished to extend or modify the model.

(1) Using hybridoma techniques, a monoclonal antibody has been developed against the glycoprotein component of the TSH receptor. This antibody blocks TSH binding and the ability of TSH to stimulate cell function but does not itself stimulate these functions. Evidence is thus provided that those antibodies in patients with Graves' disease which block TSH binding need not be thyroid stimulators causing hyperthyroidism.

(2) Additional peptides related to the α chain of cholera toxin, the α chain of TSH, and nonapeptide hormones have been synthesized. The peptides can, like the cholera toxin α subunit, stimulate adenylate cyclase activity in several membrane systems. Unlike the cholera toxin α subunit, but like TSH and nonapeptide hormones, the stimulation is not dependent on an intrinsic ADP ribosyltransferase activity. The peptides act at the G regulatory subunit level of the cyclase. Although they contain 1 mole of F^-/mole peptide, F^- does not account for the peptide action.

3) Two thyroid membrane enzymes which regulate adenylate cyclase activity have been described. Their activities are altered in vivo in functional (TSH-stimulated) and nonfunctional thyroid cells and in vitro by TSH and the peptides.

(4) Liposome reconstitution studies have shown hormone specificity analogous to the membrane and cell when the TSH interaction with the glycoprotein receptor component is measured as fluorescent dye release from the internal liposome space rather than binding.

(5) The plant lectin, concanavalin A, has been shown to regulate TSH receptor expression in vitro; an intrinsic thyroid lectin with similar properties has been detected.

(6) A new solid phase TSH radioreceptor assay, 100-fold more sensitive and significantly less costly than current assays, has been developed. Its evaluation for clinical diagnostic use is being pursued.

(7) Iodide uptake by thyroid cells in culture has been defined in terms of associated ion fluxes.

(8) Higher order gangliosides have been resynthesized in 1-8R thyroid membranes, i.e., those with a TSH receptor defect. Partial return of TSH receptor function has been evidenced.

II. Definition of the Structure and Function of the Tetanus Toxin Receptor.

Tetanus toxin has been shown to interact with thyroid plasma membranes. The properties of this interaction closely resemble the properties of the TSH interaction with TSH receptors on these membranes; the interaction of tetanus toxin with thyroid membranes exhibit several characteristics of its interaction with neural tissue; tetanus toxin does not bind to membranes of a thyroid tumor with a TSH receptor defect; and tetanus toxin stimulates thyroid hyperfunction in mice subjected to conditions known to precipitate thyroid storm. These results predicted the tetanus receptor would be similarly structured to the TSH receptor and that tetanus toxin and TSH might have common functional elements in message transmission. This hypothesis has been extended.

(1) Dissociated rat embryo cerebral cells can be maintained in monolayer cultures in the presence of a mixture of the same six hormones used for thyroid cells. These conditions permit expression of neuronal characteristics and prevent glia proliferation.

(2) The neural cells incorporate ^{125}I-tetanus in a time and ligand concentration-dependent fashion and with properties of a receptor-mediated process. Uptake can be partially prevented by unlabeled toxin or by a goat antitoxin. Pulse-chase studies in which radiolabeled toxin is exposed to cells and then chased with unlabeled tetanus toxin, tetanus toxoid, or tetanus antitoxin indicate that after 2 hours radioactivity released in the medium approaches similar steady-state levels irrespective of the compound added. This finding suggests a receptor-mediated process for uptake. The nonchased radioactivity associated with the cells can be completely extracted by sequential treatment with acetic acid or NaOH. In contrast, only about 25% to 35% of the radioactivity associated with the cells after chase can be extracted by either Nonidet P-40 or Triton X-100; the bioactivity of this detergent-extracted fraction is entirely preserved. The localization of the detergent-resistant toxin has been monitored by indirect immunofluorescence techniques. Preliminary evidence suggests that it is associated with the cytoskeleton proteins.

Properties of the retrograde axonal transport of tetanus toxin have been further defined. Gangliosides and the glycoprotein component can inhibit 95% of uptake with no loss in toxicity. Two toxin binding components are evident in this in vivo study.

III. Structure and Function of the Interferon Receptor.

(1) The glycoprotein fragments of the interferon receptor which enhance interferon bioactivity in vivo 10- to 100-fold have been further defined.

(2) TSH has been shown to have antiviral action; interferon has been shown to affect thyroid cells both with respect to antiviral protection and iodide transport.

(3) Interferon effects on ATP levels of cells and the effect of NH_4Cl on interferon bioactivity, ATP levels, and internal proton concentration have been related.

IV. Thyroglobulin (Tg) Interactions with Thyroid Membranes.

Thyroglobulin has been shown to interact with thyroid membranes.

(1) There is a receptor recognizing N-acetylglucosamine residues on the Tg molecule. Its function has been associated with the iodination process. The receptor is a glycoprotein whose activity can be reconstituted in liposomes.

(2) There is a sugar-PO_4 residue on thyroglobulin. This moiety may be important in both the functional integrity of the Tg molecule and in the targeting to the lysosome during Tg processing to T_3/T_4.

(3) The receptor assays have defined abnormal Tg species in chickens with genetic organ-specific autoimmune thyroid disease and euthyroid Graves' disease.

(4) Thyroglobulin present in the sera of patients is a better inhibitor of ^{125}I-Tg binding in the receptor assay than normally purified Tg. It also can inhibit thyroid function in membranes and cells.

Significance to Biomedical Research and the Progress of the Institute:

As outlined in the "Objectives" section, the work is directly applicable to the pathophysiology of Graves' disease and the mechanism of action of cholera and tetanus toxin. In regard to medicinal chemistry, relationships have been defined which hold new opportunities for the development of peptide analogs which will mimic the action of effectors regulating body functions (glycoprotein hormones) and blocking viral infestation of the cell (interferon). The development of agents to enhance or mimic interferon activity has been initiated. These agents may be important in preventing or reversing autoimmune diseases or the oncogenic state. A primary thyroid lesion has been defined in autoimmune thyroid disease. Monoclonal antibodies and a new solid phase receptor assay have been defined which may serve as a clinical assay of primary receptor abnormalities in hyperthyroid or hypothyroid states.

Proposed Course:

The structure of glycoprotein hormone receptors and the mechanism of action of

glycoprotein hormones will be further clarified. The basis for hormone- and toxin-directed transport in mammalian cells will be further defined, and studies on thyroid cell transport will be continued. The mechanism of interferon interaction with cell membranes will be further defined as will the relationship of the glycoprotein hormones to the following bacterial toxins: cholera toxin, botulinus toxin, tetanus toxin, and various enterotoxins produced by gram-negative bacteria. The relationships and implications to Graves' disease, exophthalmos, thyroid storm, tetanus, cholera, botulism, etc., will be pursued.

Publications:

Aloj, S. M., Lee, G., Consiglio, E., Formisano, S., Minton, A. P., and Kohn, L. D.: Dansylated thyrotropin as a probe of hormone-receptor interactions. J. Biol. Chem. 254: 9030-9039, 1979.

Aloj, S. M., Lee, G., Grollman, E. F., Beguinot, F., Consiglio, E., and Kohn, L. D.: Role of phospholipids in the structure and function of the thyrotropin receptors. J. Biol. Chem. 254: 9040-9049, 1979.

Consiglio, E., Salvatore, G., Rall, J. E., and Kohn, L. D.: Thyroglobulin interactions with thyroid plasma membranes: the existence of specific receptors and their potential role. J. Biol. Chem. 254: 5065-5076, 1979.

De Wolf, M. J. S., Consiglio, E., Winand, R. J., and Kohn, L. D.: Autoimmunity, the thyrotropin receptor plus exophthalmos. In Klein, E., Horster, F. A., and Beysel, D. (Eds.): Proceedings of the International Conference on Thyroid Autoimmunity, Munich, West Germany. Stuttgart and New York, F. K. Schattauer-Verlag, 1979, pp. 125-147.

De Wolf, M. J. S., Yavin, E., Yavin, Z., Consiglio, E., Vitti, P., Shifrin, S., Epstein, M., Gill, D. L., Grollman, E. F., Lee, G., and Kohn, L. D.: Studies on the mechanism of action of thyrotropin. Proceedings of the International Symposium on Recent Progress of Hormones, Proteins, and Enzymes, Gardone, Riviera, France, 1980, Excerpta Medica, Amsterdam, Elsevier-North Holland, in press.

Epstein, M., Ross, E. M., De Wolf, M. J. S., Fridkin, M., and Kohn, L. D.: Glycoprotein hormone--cholera toxin relationships: implications for role of ADP-ribosylation in cyclase activation. In Sugimura, T., and Smulson, M. (Eds.): Novel ADP-Ribosylations of Regulatory Enzymes and Proteins. New York, Elsevier-North Holland, in press.

Gill, D. L., Grollman, E. F., and Kohn, L. D.: Calcium transport mechanisms in membrane vesicles from guinea pig brain synaptosomes. J. Biol. Chem. 255, in press.

Grollman, E. F., Consiglio, E., Aloj, S. M., Meldolesi, M. F., and Kohn, L. D.: Structure : function studies of thyrotropin (TSH) and interferon receptors: potential role of gangliosides. Proceedings of the International Congress on Cell Biology, New York, John Wiley & Sons, 1980, in press.

Habig, W. H., Hardegree, M. S., Grollman, E. F., Lee, G., Ramos, S., and Kohn, L. D.: Interaction of tetanus toxin with thyroid membranes. Proceedings of the 5th International Tetanus Conference, Stockholm, Sweden, June 18-23, 1978, in press.

Habig, W. H., Hardegree, M. C., and Kohn, L. D.: Tetanus toxin: structure-function relationships. Proceedings of the International Symposium on Frontiers in Protein Chemistry, Honolulu, Hawaii, July 2-6, 1979, in press.

Hill, I. R., Levin, I. W., Lazo, P. S., and Kohn, L. D.: Effect of ganglioside and ganglioside-cholera toxin interactions on the vibrational Raman spectra of the bilayer lipid in dipalmitoyl phosphatidylcholine liposomes. Biochemistry, in press.

Kohn, L. D., Consiglio, E., De Wolf, M. J. S., Grollman, E. F., Ledley, F. D., Lee, G., and Morris, N. P.: Thyrotropin receptors and gangliosides. In Dreyfus, H., Mandel, P., Svennerholm, F., and Urban, D. F. (Eds.): Proceedings of the International CNRS Symposium on Structure and Function of Gangliosides, Bischenberg (Strasbourg), France, April 23-27, 1979. New York, Plenum Publishing Corp., in press.

Maheshwari, R. K., Lazo, P. S., Friedman, R. M., and Kohn, L. D.: Enhancement of interferon activity by a membrane glycoprotein related to the thyrotropin receptors. Proceedings of the International Symposium on Interferon, Dallas, Texas, October 18-20, 1979, Wadley Institute of Molecular Medicine, in press.

Meldolesi, M. F., Macchia, V., Laccetti, P., and Kohn, L. D.: Alteration of the solubilized thyrotropin receptor in a thyroid tumor whose basal adenylate cyclase activity is maximally stimulated in the absence of thyrotropin or cholera toxin. Proceedings of the 8th International Thyroid Congress, Sidney, Australia, February 3-8, 1980, in press.

Morris, N. P., Consiglio, E., Kohn, L. D., Habig, W. H., Hardegree, M. C., and Helting, T. B.: Interaction of fragments B and C of tetanus toxin with neural and thyroid membranes and with gangliosides. J. Biol. Chem. 255, in press.

Ramos, S., Grollman, E. F., Lazo, P. S., Habig, W. H., Hardegree, M. C., Kaback, H. R., and Kohn, L. D.: Effect of tetanus toxin on the accumulation of a permanent lipophilic cation ($TPMP^+$) by guinea pig synaptosomes. Proc. Natl. Acad. Sci. U.S.A. 76: 4783-4787, 1979.

Winand, R. J., DeWolf, M. J. S,, Consiglio, E., and Kohn, L. D.: Autoimmunity, the thyrotropin receptor, and exophthalmos. In Klein,. E., and Horster, F. A. (Eds.): Autoimmunity in Thyroid Diseases. Stuttgart and New York, F. K. Schattauer-Verlag, 1979, pp. 125-147.

Winand, R. J., Winand-Devigne, J., Etienne-Decerf, J., Wadeleux, P. A., and Kohn, L. D.: Thyroid cytotoxic antibodies: a simple quantitative assay, their presence in thyroid diseases, and their properties. J. Clin. Endocrinol. Metab., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 23,970-05 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cancerostatic and Enzyme Regulating Agents and Intermolecular Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Koloman Laki, Ph.D. and Chief, Section on Physical Biochemistry	Research Chemist	LBP NIAMDD
OTHERS:	Terez Szabo, M.D.	Visiting Associate	LBP NIAMDD
	Judith C. Kertesz, Ph.D.	Visiting Fellow	LBP NIAMDD
	Thomas G. Burke, M.S.	Guest Worker	LBP NIAMDD
	Kornelia Farkas, M.D.	Guest Worker	LBP NIAMDD
	James E. Girard, Ph.D.	Guest Worker	LBP NIAMDD
	Laszlo Muszbek, M.D.	Guest Worker	LBP NIAMDD

COOPERATING UNITS (if any) Dr. A. K. Agrawal (PRB,NICHHD); Dr. A. Ahmed (NMRI); Dr. E. Fesus (LBC,NIAMDD); Dr. A. Mukherjee (PRB,NICHHD); Dr. S. T. Yancey (LCHPH,NCI); Departments of Internal Medicine and Central Clinical Chemistry, Medical Univ., Debrecen, Hungary; Immunology Laboratory, Navy Medical Res. Institute (NMRI)

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Physical Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.9	3.4	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The alkylisocyanates liberated from BCNU and NCNU are active-site directed inhibitors of the enzyme transglutaminase (Factor XIII in plasma). We have previously reported that these drugs keep the enzyme in the organs of the mice inactivated up to 16 hours after administration. Our explanation for the cancerostatic action of these nitrosoureas is that these compounds inactivate transglutaminase which carries out the cover-up process for the proliferating cells. It can be shown that in the cytotoxic test, release of ^{51}Cr from the targets is inhibited if fibrinogen molecules are attached to the cell surface with the help of transglutaminase. Such masked cells are even more vicious when implanted into mice and are poor targets for killer lymphocytes.

PHS-6040

Project Description:

Objectives:

I. Host versus the proliferating cell. Proliferating cells in the host organisms are considered alien, and the explanation is advanced that a "cover-up" process protects them from the host's cellular immune system. II. Nature of the peptide bond bond. ^{13}C NMR studies of N-methylacetamide show that ions of neutral salts bind to the peptide bond.

Major Findings:

I. Host Versus the Proliferating Cell

In the past several years we have been studying the role of transglutaminase in the proliferation of tumor cells in mice. Our experience is summarized with the proposition that cancer cells are able to cover-up their "strangeness" and "vulnerability" from T-lymphocytes with the use of transglutaminase (Factor XIII in blood plasma). Some of the observations that lead to the "cover-up" proposition are as follows: Antibody against transglutaminase slows down the growth of implanted cancer in mice. Intravenous administration of transglutaminase speeds up metastasis of Lewis lung tumor. Elimination of syngenic tumor after BCNU or NCNU administration follows the course of allogenic tumor elimination.

Here we report briefly that by inactivating transglutaminase with alkyl isocyanates in the tumor-bearing or pregnant mice, T-cells are generated which kill the unmasked tumor cells and the unmasked conceptus. We find that cancerostatic action of nitrosoureas can be correlated to the carbamoylation of the active site of transglutaminase. Furthermore, the carbamoylation is more pronounced with nitrosurea of which the alkylisocyanate moiety fits the active site of transglutaminase.

For these experiments we used CAF_1 mice and YPC-1 plasma cell tumors. The tumor-bearing mice were treated with BCNU or NCNU, one yielding chloroethyl isocyanate, the other neopentyl isocyanate. For the immunization, tumor cells (subcutaneous or intraperitoneal) were removed a few hours after BCNU or NCNU administration. These cells, inactivated either with radiation or mitomycin, were used in in vivo and subsequently in in vitro sensitization of the splenic lymphocytes of normal mice. These sensitized lymphocytes specifically kill, in the 4-hour cytotoxic test, ^{51}Cr-labeled tumor cells: cold targets reduce ^{51}Cr release, while E14 cells do not.

Our finding that NCNU given to CDF_1 (Balb/c x DBA) and to CAF_1 (Balb/c x AL/n) eliminates implanted L1210, P388, YPC-1, and YLL tumors days after the decomposition of the drug, supports the view that it is the immune system which kills tumor cells devoid of their protective shield. The involvement of the immune system is further demonstrated by the finding that mice "cured" with these drugs will not take up the tumor.

The role of transglutaminase in the proliferating conceptus is indicated by the finding that Factor XIII-deficient women not able to "mask" the fetus, abort it. (With the addition of Factor XIII, the pregnancy is successful.)

In cooperation with A. B. Mukherjee (PRB, NICHHD) and A. K. Agrawal (PRB, NICHHD), we find that the conceptus taken out from the tuba of pregnant mice treated with NCNU 4 hours earlier, readily develops in tissue culture to the morula or blastocyst stage, while if left in the animal it would have been destroyed. This again indicates that it is not the drug which kills the conceptus, but the immune system. Recent experiments show that indeed conceptus "unmasked" by inhibiting transglutaminase, stimulates blast transformation of lymphocytes. Evidence can be marshalled to propose that in the case of the conceptus it is uteroglobin which is attached to the cell surface by transglutaminase (Factor XIII) in order to protect the fetus from the mother's immune system.

It appears that proliferating cells are "strangers" to the host organisms and that their strangeness (altered self) is masked by transglutaminase attaching a protein to the "modified self" antigen.

Recognition and subsequent killing by T-lymphocytes involves the modified histocompability antigen (altered self) on the targets. In the proposition we are developing, the "self" modified by fetal antigens is a substrate of transglutaminase. One constitutent of the altered self is beta-2-microglobulin (β-2-m). We now find that β-2-m is a substrate of transglutaminase. By the action of the enzyme, large polymers of this small protein form; also, labeled methylamine becomes incorporated. Fluorescent antibody against β-2-m revealed that the even distribution of β-2-m on the cell surface becomes patchy after transglutaminase incubation.

II. Nature of the Peptide Bond

Interactions of ions with N-methylacetamide. ^{13}C NMR (nuclear magnetic resonance) spectroscopy is used to investigate the binding of LiBr, LiCl, LiF, NaF, and NaCl to NMA (N-methylacetamide) as a model for the ion binding to a peptide bond. The addition of neutral salts altered the cis-trans isomer ratio. While the salt-induced cis-isomerization is reversible with dilution, when methylglyoxal is added to NMA it forms a stable cis isomer. That confirms also our previous observations that methylglyoxal interacts with the peptide bond and not only with the lysine side chain. Our theoretical and experimental studies provide a possible explanation why lyophilized proteins give ESR spectrum.

Significance to Biomedical Research and the Progress of the Institute:

If our observations on cancer-bearing mice applies to humans, then three conditions would exist: (1) the strangeness of the tumor cell is covered; (2) the "vulnerability" is covered; or (3) both conditions are present. To determine which of these conditions exist, two new tests are recommended: (i)

test whether after NCNU incubation, tumor cells stimulate lymphocyte blast transformation or (ii) whether after NCNU incubation tumor cells become targets to the patient's lymphocytes.

Proposed Course:

We are planning to see if such tests are feasible. If so, the physican can adapt medication according to whether the strangeness or vulnerability of the tumor cells is present.

Publications:

Abdulnur, S. F., and Laki, K.: Complete neglect of differential overlap study of the binding of salts to N-methylacetamide. Biophys. J. 28: 503-509, 1979.

Damjanovich, S., Kertesz, J. C., Szabo, G., Papp, S., and Laki, K.: Methylglyoxal regulating gene transcription in vivo. Proc. Natl. Found. Cancer Res., in press.

Kertesz, J. C., and Laki, K.: Effect of neutral salts and methylglyoxal on the cis-trans equilibrium of N-methylacetamide: a 13-carbon nuclear magnetic resonance study. Int. J. Quantum Chem., in press.

Laki, K., Suhai, S., and Kertesz, J. C.: Energy bands and charge transfer in proteins. In Submolecular Biology and Cancer, Ciba Foundation Series 67. New York, Elsevier/North Holland, 1979, pp. 33-50.

Mukherjee, A. B., Laki, K., and Agrawal, A. K.: Possible mechanism of success of an allotransplantation in nature: mammalian pregnancy. Nature, in press.

Szabo, G., Kertesz, J. C., and Laki, K.: Interaction of methylglyoxal with poly-L-lysine. Biomaterials 1: 27-29, 1980.

Tarantino, A., Thompson, P., and Laki, K.: The reaction of the cancerostatic CCNU with the catalytic site of transglutaminase. Cancer Biochem. Biophys. 4: 33-36, 1979.

Honors:

Dr. Koloman Laki was appointed Senior Research Professor (Honorary), Department of Chemistry, American University, Washington, D.C.

Dr. Laki was appointed a Member of the Editorial Board of Biomaterials.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,020-17 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cell Surfaces: (a) Phagocytosis and Adhesion in Macrophages (b) E. coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Loretta Leive, Ph.D.	Research Biologist	LBP NIAMDD
OTHERS:	Robert C. Goldman, Ph.D.	Staff Fellow	LBP NIAMDD
	Seiichi Takasaki, Ph.D.	Visiting Fellow	LBP NIAMDD
	Yoshiko Takasaki, M.A.	Guest Worker	LBP NIAMDD

COOPERATING UNITS (if any)

None

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SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.3	4.0	1.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the properties and structure of the outer membrane of E. coli. A mutant showing increased permeability to a large number of hydrophobic compounds was found before to have altered lipopolysaccharide and no alteration in other outer membrane components. We now have shown that the altered lipopolysaccharide increases the fluorescence of hybrophobic tetracycline derivatives more than normal lipopolysaccharide, suggesting that the altered lipopolysaccharide permits increased permeability by increasing partition of hydrophobic molecules into the membrane. We have also devised a gel electrophoresis method that shows far more heterogeneity in lipopolysaccharide from a single strain than heretofore detected. In other experiments we find that the small amount of protein that copurifies with lipopolysacchride in butanol "endotoxin" preparations is all outer membrane protein which is of interest because of the lymphocyte mitogenicity of this protein.

In studies with macrophage cell lines, we have selected mutants that do not show generalized phagocytosis. We also have defined by gel electrophoresis several external membrane proteins that are reduced by borotritide after sialic acid oxidation, and that completely lose their label on sialidase treatment. These will be used as markers in studies of membrane dynamics.

Project Description:

The Outer Membrane of E. coli

The outer membrane permits small hydrophilic molecules to enter through pores but excludes hydrophobic molecules, including antibiotics, drugs, and detergents. Its protein, lipid, and lipopolysaccharide (LPS) composition is known, but their spatial arrangement, the precise structure of LPS, and the mechanism of the exclusion of hydrophobic molecules, is still under investigation.

We have previously isolated the first known mutant that, along with increased permeability to hydrophobic compounds, shows changes solely in LPS. We now show that a hydrophobic tetracycline derivative fluoresces more when added to cells of the mutant than when added to the wild type, suggesting that the mutation permits increased membrane association of this drug. When added to purified LPS, this drug also fluoresces more than when added to wild type LPS. This result supports the hypothesis that the change in LPS directly causes the permeability change by permitting greater partition of this molecule into the LPS of the outer membrane.

Previous studies in this and other laboratories suggested that the polysaccharide of LPS may contain one to at most four size classes in a given strain. Using sodium dodecylsulfate gel electrophoresis, we now can detect up to 40 different lengths of LPS in E. coli and Salmonella typhimurium strains thought previously to have one or two. Each consecutive band represents addition of one or more repeating pentasaccharide antigenic side chain unit to the lipid-core region, as shown by measuring the ratio of radioactivity in side chains to radioactivity in phosphate of the core and lipid.

We earlier devised a method for extracting LPS from whole cells with aqueous butanol resulting in a product that was 90-98% LPS plus 2-10% protein. This material received a great deal of attention from other, immunological, laboratories, as the small amount of protein that copurifies and is pronase-resistant is mitogenic for mutant mice that do not respond to LPS as a mitogen. We have now shown that the proteins that copurify are specifically outer membrane proteins and have proven, with the aid of specific antibody, the identity of two of the four pronase-resistant proteins.

Macrophage Mutants and Cell Function

We have selected mutants of a macrophage cell line that do not show generalized phagocytosis, using their ability to survive addition to the medium of beads coupled to toxic molecules as a selective mechanism. Two stable mutants have been selected so far. These mutants will be studied to determine pathways involved in phagocytosis.

We have labeled membranes of macrophage cell lines by borotritide reduction of oxidized sialic acid residues, and defined several labeled protein bands, on sodium dodecylsulfate gels, that completely lose their label on sialidase

treatment. These proteins are being used as markers in studies of the specificity of internalization, and rate of entry and recycling, of membrane markers during phagocytosis and pinocytosis.

Derivatives of S. typhimurium, differing only in their LPS side chain composition, were shown by Dr. Makela to vary one thousand-fold in their lethality for mice. In vivo clearance data suggested a role for phagocytosis in this difference. We now show that macrophage cell lines phagocytose the least toxic organism much more rapidly than the most toxic one. The possible role of the macrophage mannose receptor and other receptors in this process is being explored. These studies are being due in collaboration with Dr. P. Helena Makela of the Central Public Health Laboratory, Helsinki, Finland.

Publications:

Coleman, W. G., Jr., and Leive, L.: Two mutations which affect the barrier function of the Escherichia coli K-12 outer membrane. J. Bacteriol. 139: 899-910, 1979.

Goldman, R. C., and Leive, L.: Heterogeneity of antigenic side chain length in lipopolysaccharide from Escherichia coli 0111 and Salmonella typhimurium LT2. Eur. J. Biochem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,120-09 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies in Traumatic Shock and Cellular Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Sanford M. Rosenthal, M.D. Scientist Emeritus LBP NIAMDD

COOPERATING UNITS (if any)

None

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Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effect of sodium chloride on longevity is a long-term study, and results will be forthcoming.

PHS-6040

Project Description

Objectives:

To investigate the effect of NaCl in the diet of mice on extravascular fluid movement and longevity.

Methods Employed:

Swelling of the mouse tail was measured on application of a mild vacuum to an inbred strain of mice (AIJ). Diets were prepared containing 0.5% to 4.5% NaCl. An apparatus for measuring blood pressure in mice is being assembled.

Major Findings:

The effect of NaCl on longevity is a long-term study, and results will be forthcoming.

Proposed Course:

To determine the effect of expansion of the extracellular fluid volume on the microcirculation and longevity.

Publications:

Markley, K.: Burned patients and immunoregulation. J. Trauma 19, Suppl.: 891-892, 1979.

Markley, K., Smallman, E. T., and Briggs, L. W.: Early mortality and temperature regulation in burned mice following administration of catecholamines and adrenergic receptor blocking drugs. J. Trauma 19: 512-519, 1979.

Rosenthal, S. M.: In memorium, Kehl Markley (1924-1979). In Proceedings of the Conference on Immune Consequences of Thermal Injury, Lake Arrowhead, California, December 2-5, 1979. Baltimore, Williams & Wilkins, 1980, in press.

Rosenthal, S. M.: Physiological changes with age in the microcirculation and lymphatics. In Masuro, E. J. (Ed.): CRC Handbook of Physiological Changes with Age. Cleveland, Ohio, The Chemical Rubber Co., in press.

Rosenthal, S. M.: The treatment of shock. J. Trauma 19, Suppl.: 875-876, 1979.

Honors:

Dr. Sanford M. Rosenthal was given the 1980 ASPET Award for Experimental Therapeutics on April 16, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,140-14 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Edith W. Miles, Ph.D. Research Chemist LBP NIAMDD

COOPERATING UNITS (if any)

None.

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Laboratory of Biochemical Pharmacology

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Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure, function, and subunit interaction in the multienzyme complex tryptophan synthase are being investigated. We have determined the primary sequence of the first 99 residues of the β_2 subunit and located 3 active site residues therein: cysteine-62, lysine-87, and histidine-86. Two other important cysteine residues have been shown to be cysteine-170 and cysteine-230.

The function of tryptophan synthase has been probed by spectroscopic studies of the interaction between the cofactor, pyridoxal phosphate, and certain amino acid substrates, products, and substrate analogs. An intermediate formed during the reaction of L-serine with enzyme-bound [^{3}H]pyridoxal phosphate has been reduced by $NaBH_4$ and isolated.

The interaction between the α and β_2 subunits of tryptophan synthase has been studied by ultraviolet difference spectroscopy and by studies using limited tryptic proteolysis. Two proteolytic fragments of the α subunit have been isolated and shown to refold independently and reassociate to give active enzyme.

PHS-6040

Project Description:

Objectives:

Work in our laboratory is aimed at elucidating the structure, function, and subunit interaction in the Escherichia coli tryptophan synthase $\alpha_2\beta_2$ complex. This complex is a simple model of more complicated multienzyme complexes. Specific goals are the determination of how the α and β subunits are arranged in the complex, how the active sites of the α and β subunits are arranged to form a composite active site, and how subunit interaction changes the rates and specificities of the reactions catalyzed at the active site of each subunit. One specific advantage of this system for achieving these goals is the presence of a chromophoric cofactor at the active site of the β subunit. The formation of pyridoxal phosphate substrate intermediates can be used to follow kinetics and elucidate detailed mechanisms. The change in environment of pyridoxal phosphate upon subunit association can be used as a probe of subunit association and the composite active site. Our goal of determining the chemistry of residues in the active site of each subunit is being achieved by the use of specific chemical modification reagents followed by primary sequence analysis of the modified protein chains.

Methods Employed:

Crystalline α and β_2 subunits and $\alpha_2\beta_2$ complex of tryptophan synthase are prepared and assayed by methods previously developed in this laboratory. Absorbance spectra and difference absorbance spectra are made with a Cary 118 spectrophotometer. Circular dichroism spectra and fluorescence spectra are also made. Subunit association studies are made using gel filtration. Chemical modification studies are carried out using N-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid), 2-nitro-5-thiocyanobenzoic acid, iodoacetic acid, and the substrate analog L-2-amino-4-methoxy-trans-3-butenoic acid. Affinity labeling is carried out using 4-bromoacetylpyridoxamine 5'-phosphate, which has been synthesized in our laboratory. Modified enzymes are examined for their ability to combine with pyridoxal 5'-phosphate or with α subunit and for their ability to carry out partial reactions. Modified amino acid residues and peptides are identified by labeling with radioactive compounds and by techniques of proteolytic digestions, peptide mapping, ion exchange chromatography, electrophoresis, gel filtration, disc gel electrophoresis, thin-layer chromatography, nuclear magnetic resonance, mass spectrometry, infrared spectrometry, amino acid analysis, and protein sequencing. Limited tryptic hydrolysis and isolation of protein fragments are also carried out. Enzyme-substrate intermediates with [^{3}H]pyridoxal phosphate bound to the $\alpha_2\beta_2$ complex are reduced with $NaBH_4$ and isolated for stereochemical analysis.

Major Findings:

The structure, function, and subunit interaction in the multienzyme complex tryptophan synthase are being investigated. We have determined the primary sequence of the first 99 residues of the β_2 subunit and located 3 active site residues therein: cysteine-62, lysine-87, and histidine-86. Two

other important cysteine residues have been shown to be cysteine-170 and cysteine-230.

The function of tryptophan synthase has been probed by spectroscopic studies of the interaction between the cofactor, pyridoxal phosphate, and certain amino acid substrates, products, and substrate analogs. An intermediate formed during the reaction of L-serine with enzyme-bound [^{3}H]pyridoxal phosphate has been reduced by $NaBH_4$ and isolated.

The interaction between the α and β_2 subunits of tryptophan synthase has been studied by ultraviolet difference spectroscopy and by studies using limited tryptic proteolysis. Two proteolytic fragments of the α subunit have been isolated and shown to refold independently and reassociate to give active enzyme.

Publications:

Higgins, W., Fairwell, T., and Miles, E. W.: An active proteolytic derivative of the α subunit of tryptophan synthase. Identification of the site of cleavage and characterization of the fragments. Biochemistry 18: 4827-4853, 1979.

Higgins, W., Miles, E. W., and Fairwell, T.: Location of three active site residues in the NH_2-terminal sequence of the β_2 subunit of tryptophan synthase from Escherichia coli. J. Biol. Chem. 255: 512-517, 1980.

Miles, E. W.: Tryptophan synthase: structure, function, and interaction with D-tryptophan and L-tryptophan. Proceedings of the 3rd International Meeting of the International Study Group for Tryptophan Research, Kyoto, Japan, August 4-7, 1980. New Amsterdam, The Netherlands, Elsevier-North Holland Biomedical Press, in press.

Miles, E. W., and Higgins, W.: Location of the reactive sulfhydryls in the primary sequence of the β_2 subunit of tryptophan synthase of Escherichia coli. Biochem. Biophys. Res. Commun. 93: 1152-1159, 1980.

Honors:

Invited participant and speaker at the 3rd International Meeting of the International Study Group for Tryptophan Research, August 4-7, 1980, Kyoto, Japan.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,150-09 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Intermolecular Interactions in Physical Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Allen P. Minton, Ph.D.	Research Chemist	LBP NIAMDD
OTHER:	Jacob Wilf, Ph.D.	Visiting Fellow	LBP NIAMDD

COOPERATING UNITS (if any)

Dr. Marc S. Lewis, Biomedical Engineering and Instrumentation Branch, Division of Research Services, NIH

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.9	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A comprehensive theoretical study of excluded volume effects upon biochemical equilibria and kinetics has been carried out. The self-association of myoglobin in concentrated solution and in the presence of high concentrations of globular proteins has been studied by measurement of sedimentation equilibrium and fluorescence polarization. The effect of thermodynamic nonideality upon the partition coefficient measured in exclusion chromatography has been theoretically studied. A quantitative model for hormone action has been formulated and applied to the analysis of experimental data.

Project Description:

Objectives:

(1) To explore the effect of excluded volume upon the equilibria and kinetics of biochemical reactions. (2) To explore the relationship between the binding of pharmacologically active ligands (hormones, drugs, lectins) to the cell surface and the magnitude of the biological response elicited by the ligand.

Methods Employed:

Theoretical studies employ methods of classical and statistical thermodynamics and formulations of complex multiple equilibria, computer techniques for fitting theoretical model functions to experimental data, and computer techniques for numerical solution of problems involving multiple equilibria. Experimental studies utilize measurements of sedimentation equilibrium, fluorescence intensity and polarization, and standard preparative and analytical methods in protein chemistry.

Major Findings:

(1) A comprehensive theoretical study of excluded volume effects upon biochemical equilibria and kinetics has been carried out. Several previously unobserved phenomena are predicted to occur in solution as the volume fraction of globular macromolecules increases: (a) Compact quasispherical macromolecular conformations become increasingly energetically favored over extended anisometric conformations. (b) Self- and hetero-association processes are enhanced, particularly those leading to the formation of compact quasispherical aggregates. (c) The native conformation of a protein becomes more stable relative to any less compact denatured conformation. (d) Depending upon the details of the particular reaction mechanism, the rate of an enzyme-catalyzed reaction may monotonically decrease, go through a maximum, or exhibit more complex behavior. (A. P. Minton)

(2) According to the study referred to in item (1) above, myoglobin is predicted to self-associate in sufficiently concentrated solution. Equilibrium sedimentation experiments have been carried out on highly concentrated solutions of hemoglobin and myoglobin (up to over 30 g/dl) using specially designed ultracentrifuge cells with short light paths (0.1-0.2 mm). Preliminary analysis of the data indicates the occurrence of self-association of myoglobin at high concentration. [A. P. Minton and M. S. Lewis (BEIB, DRS)]

(3) According to the study referred to in item (1) above, myoglobin at low concentration is predicted to self-associate in the presence of high concentration of other globular proteins. The effectiveness of the "background" protein in inducing myoglobin self-association is predicted to decrease with increasing molecular weight. Measurement of the fluorescence polarization of fluorescent derivatives of myoglobin and hemoglobin as a function of the concentration of added protein or random-coil polymer indicates that

myoglobin self-associates to form a hemoglobin-like oligomer in the presence of high concentrations of small proteins (lysozyme, ribonuclease A, β-lactoglobulin) but does not self-associate in the presence of the polymers PEG-6,000 and PEG-20,000, or the larger protein ovalbumin. These results are in qualitative accord with predictions of the theory. (J. Wilf and A. P. Minton)

(4) The dependence of the partition coefficient in exclusion chromatography upon concentration of macromolecular solute has been theoretically studied for highly non-ideal solutions of both nonassociating and self-associating solutes. It was found that exclusion chromatographic data which were interpreted by other investigators as providing evidence for the self-association of hemoglobin at high concentrations may be quantitatively accounted for by the new theory without invoking self-association of hemoglobin. (A. P. Minton)

(5) A quantitative model has been formulated for the mechanism of action of certain types of hormones. According to this model a single hormone molecule is treated as bearing two distinct regions capable of simultaneous interaction with the cell surface: a specificity determinant which binds with high affinity and specificity to one class of cell surface sites called receptor, and a response determinant which binds with much lower affinity and specificity to a second class of cell surface sites called effector. It is postulated that the steady-state response elicited by the hormone is proportional to the amount of hormone bound to effector, and that under ordinary conditions, the ratio of effector sites to receptor sites is substantially less than unity, perhaps on the order of 0.1. In this model, the formation of hormone-receptor complex does not serve to directly initiate the process by which response is elicited, but rather to selectively increase the apparent affinity of effector for a specific hormone by several orders of magnitude. (A. P. Minton)

Proposed Course:

Projects (2) and (3) above should be completed within the next several months. A collaborative study (with Dr. William M. Egan, Division of Bacterial Products, Bureau of Biologics) is planned wherein we will attempt to employ a new NMR technique developed by Dr. Egan and collaborators to measure rotational relaxation times of proteins labeled with ^{13}C in the presence of high concentrations of other (unlabeled) proteins. A collaborative study (with Dr. Philip D. Ross, Laboratory of Molecular Biology, NIAMDD) is planned wherein we will explore the utility of viscosity measurements as a tool for studying self-association in highly concentrated protein solutions. Kinetic properties of the bivalent ligand model for hormone action will be explored.

Significance to Biomedical Research and the Program of the Institute:

Theoretical and experimental studies of the behavior of proteins and other macromolecules in volume-occupied solutions represent a first attempt to ascertain the differences which are likely to exist between biochemical reactions in dilute solution (typical experimental conditions in the laboratory) and the same reactions in physiological media.

The new theoretical analysis of results obtained from exclusion chromatographic experiments on highly nonideal solutions enhances the value of this method as a tool for the study of weakly associating macromolecules.

The bivalent ligand model for hormone action is capable of qualitatively and semiquantitatively rationalizing a broad variety of experimental data on the relationship between hormone binding and steady-state response elicitation, both in the absence and presence of cross-linking agents such as anti-hormone antibodies, lectins, and anti-membrane-protein antibodies.

Publications:

Aloj, S. M., Lee, G., Consiglio, E., Formisano, S., Minton, A. P., and Kohn, L. D.: Dansylated thyrotropin as a probe of hormone-receptor interactions. J. Biol. Chem. 254: 9030-9039, 1979.

Minton, A. P.: On the interpretation of binding isotherms in complex biological systems: apparent homogeneity of some heterogeneous systems. Biochim. Biophys. Acta 558: 179-186, 1979.

Minton, A. P.: Thermodynamic nonideality and the dependence of partition coefficient upon solute concentration in exclusion chromatography. Biophys. Chem., in press.

Pumphrey, J. G., Holroyd, K., and Minton, A. P.: Oxygen equilibrium of emulsified solutions of normal and sickle hemoglobin. Biochem. Biophys. Res. Commun. 88: 980-987, 1979.

Ross, P. D., and Minton, A. P.: The effect of non-aggregating proteins upon the gelation of sickle cell hemoglobin: model calculations and data analysis. Biochem. Biophys. Res. Commun. 88: 1308-1314, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,260-14 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Enzymatic Studies of Nucleic Acid Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nancy G. Nossal, Ph.D.	Research Chemist	LBP NIAMDD
OTHERS:	Lynn L. Silver, Ph.D.	Staff Fellow	LBP NIAMDD
	Malabi Venkatesan, Ph.D.	Visiting Fellow	LBP NIAMDD

COOPERATING UNITS (if any)
Drs. A. Roth and P. T. Englund, Johns Hopkins University, Baltimore, Maryland, and Drs. K. Williams, E. Spicer, and W. Konigsberg, Yale University, New Haven, Connecticut

LAB/BRANCH
Laboratory of Biochemical Pharmacology

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Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	3.0	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the reactions required for DNA replication *in vitro* using proteins encoded by T4 bacteriophage. With single-stranded ϕX174 DNA as a model template, we find that a heterogeneous family of pentaribonucleotides with the sequence pppACNNN are made by the T4 gene 41 protein and a second protein controlled by gene 61. These primers are elongated to form duplex circular molecules by the gene 43 DNA polymerase, gene 45 protein, and the complex of gene 44 and 62 proteins (a DNA-dependent ATPase). If the gene 32 DNA binding protein is also present, the DNA is further elongated by strand displacement synthesis yielding rolling circles with long tails. The priming activity associated with gene 61 copurifies with a basic protein which has a native and denatured molecular weight of about 42,000. We are searching *in vivo* and *in vitro* for T4 proteins required for the initiation of DNA replication within duplex templates. We have identified and partially purified a nuclease controlled by a late T4 protein which hydrolyzes T7 and λ DNA to a series of discrete sized products.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To determine the characteristics and functions of enzymes which are involved in the synthesis, modification, and degradation of DNA.

Major Findings and Proposed Course:

We are using the bacteriophage T4 DNA replication proteins to study the reactions required for the replication of duplex DNA. We have chosen this system since T4 phage appears to induce the synthesis of the enzymes required for its own DNA synthesis, rather than depending on host proteins. Phage-infected bacteria can be grown in quantities allowing the purification of sufficient enzymes for biochemical and physical studies.

Reactions Catalyzed by the T4 Replication Proteins — Genetic studies by Edgar and Epstein et al. have shown that the proteins encoded by the phage genes 32, 41, 43, 44, 45, and 62 are required for DNA synthesis in vivo, in addition to other enzymes involved in the synthesis of DNA precursors. DNA synthesis is delayed in the absence of the gene 39, 52, 59, and 61 products. We have previously developed procedures for the purification of the gene 32 DNA binding protein; the gene 43 DNA polymerase which also has 3' to 5' exonuclease activity; the complex of the gene 44 and 62 proteins, a DNA-dependent rATP or dATPase; the gene 45 protein which stimulates the 44-62 ATPase; the gene 41 protein which has DNA-dependent nucleotidase activity; and an additional protein controlled by T4 gene 61.

Using these purified proteins we have tried to reconstruct in vitro the reactions which would be required at a replication fork. The replication of a linear duplex DNA like that of T4 is likely to involve first the recognition of one or more origins for the initiation of the synthesis of the leading strand, followed by elongation of this leading strand and the unwinding of the parental strand complementary to the template for the leading strand. The unwound parental strand in turn serves as the template for the initiation and elongation of the daughter lagging strand.

Leading Strand Synthesis — Strand displacement synthesis which occurs by the addition of nucleotides to the 3' hydroxyl group at a nick in duplex DNA resembles the elongation of the leading strand in its requirement for the simultaneous unwinding of the complementary strand. We have previously shown that T4 DNA polymerase (gene 43 protein) is unable to catalyze this reaction alone, and even in the presence of the gene 32 helix-destabilizing protein there is only a very limited displacement reaction yielding a product whose rapid renatuability suggests that the polymerase frequently switches to copy the unwound strand. Efficient strand displacement synthesis without strand switching occurs only when the 44-62 and 45 proteins are added in addition to the polymerase and 32 proteins. An rATP stimulation of this reaction can be demonstrated when the dATP concentration is limited. The product of this

five-protein reaction contains long single strands attached to the duplex template.

We are now trying to determine the role of each of these proteins in the unwinding of the complementary strand. On single-stranded templates the 44-62 and 45 proteins appear to increase the rate of synthesis by making the polymerase more processive, while the 32 protein appears to stimulate by decreasing the stability of hairpins in the template. The 32 protein could facilitate the reaction on duplex templates by destabilizing the helix ahead of the polymerase, as well as by binding to the displaced single strand. The 44-62 and 45 proteins may allow the polymerase to take advantage of the 32 protein destabilization by keeping the polymerase attached to the template, or they could convert the polymerase to a form which can unwind a duplex by a reaction analogous to that catalyzed by E. coli pol I or the T5 DNA polymerase. We have so far been unable to demonstrate the separation of the strands of nicked col E1 DNA molecules by any combination of the polymerase, 44-62, 45, and 32 proteins when assayed under conditions where DNA synthesis is prevented by the omission of one of the required dNTP. We are now constructing partial duplex molecules by annealing radioactive separated restriction fragments of ϕX Rf I to the circular viral strand. We plan to use these molecules to test for helix destabilization by the T4 proteins by assaying for the release of restriction fragments of different lengths and base compositions. If intact fragments are not released, we will look for partial destabilization by measuring increased degradation of the annealed fragments by nucleases specific for single strands. The unwinding will be measured under conditions allowing, or preventing, simultaneous DNA synthesis.

In order to test the idea that the 44-62 and 45 proteins stimulate DNA synthesis by strengthening the binding of the polymerase to DNA, we have measured the binding of the polymerase to the single-stranded circular DNA of $\phi X174$. We find that the polymerase binds weakly to this molecule which lacks a 3' OH primer site, and that the binding is only slightly increased by the 44-62 and 45 proteins, which are themselves not bound to the DNA to a measurable extent. We plan to extend these studies using the partially duplex templates discussed above. This will allow us to compare the binding of the polymerase alone, or with the 44-62, 45, and 32 proteins, to the template strand, and to a 3' OH at the end of a single primer, as well as to a 3' OH at a nick formed by the annealing of two contiguous restriction fragments. (M. Venkatesan and N. G. Nossal)

RNA Primers — The discontinuous synthesis on the lagging strand requires the repeated initiation of new DNA chains on the portions of the complementary strand which are unwound as a result of the elongation of the leading strand. T4 DNA polymerase, like all other DNA polymerases studied to date, cannot initiate new DNA chains. We have previously shown that DNA synthesis on templates without a primer, such as circular single-stranded $\phi X174$ DNA, required the gene 41 protein and a protein whose activity depended on gene 61, in addition to the 43, 44-62, 45, and 32 proteins, and was completely dependent on ribonucleotide triphosphates. The 41 and 61 proteins were shown to synthesize

a heterogeneous group of pentanucleotides whose predominant sequence is $pppAC(N)_3$, where N in positions 3 to 5 can be G, U, C, or A. These ribo-pentanucleotides were shown to function as primers for DNA synthesis and were found at the 5' terminii of DNA product chains beginning at multiple discrete positions on the DNA template.

61-Protein — During the past year we have extensively purified the gene 61 activity and have shown that this activity copurifies with a protein with a denatured molecular weight of 42,000. In the most purified fraction, the 42,000 molecular weight band represented approximately 80% of the protein present. The native molecular weight of the 61 protein was determined to be 45,000 by gel filtration on Sephadex G-150. The 61 activity behaves as a basic protein upon electrophoresis in 0.8% agarose gel at pH 7.8, migrating toward the cathode.

The protein, whose activity requires a functioning T4 gene 61 protein, was shown to be a phage protein rather than a host protein modified by the gene 61 protein. A 42,000 molecular weight protein, labeled by the addition of [^{14}C]-amino acids following T4 infection, cochromatographed with the enzymatic activity during DNA cellulose fractionation of 61^+ extracts, and was missing after chromatography of 61^- extracts. We conclude that the active form of the 61 gene product is a monomer with a molecular weight of 42,000. At the present stage of purification, we cannot eliminate the possibility that proteins in addition to the 61 gene product are required for the "61 activity."

Primer Synthesis — We have been attempting to analyze the roles of the 41 and 61 proteins in the synthesis of ribonucleotide primers by determining what, if any, partial reactions are carried out by each of the proteins separately. As yet, no positive results have been obtained. Since the pentanucleotide primer sequence is pppApCpXpXpX, we asked whether either protein was capable of forming pppApC from ATP + CTP; neither alone was competent to do so. We asked further whether either protein (or both) could use ApC as an acceptor for GTP to form ApCpG; ApCpG is not formed in detectable amounts with either or both 41 and 61, nor can ApC or ApCpC substitute for either protein or for ribotriphosphates in DNA synthesis on single-stranded DNA templates (in the presence of the 43, 44, 62, 45, and 32 proteins). 61 protein binds tightly to single-stranded DNA, but 41 protein does not. We have been unable to detect a complex of the 41 and 61 proteins.

We have recently found that primer synthesis by the 41 and 61 proteins is markedly stimulated by the 32 protein, if the 61 protein is present at high concentration. The same pentanucleotide products are formed with or without the 32 protein. We are currently trying to determine whether 32 protein increases the binding of 61 protein to sites on the DNA serving as templates for the primer, or whether it facilitates the removal of completed primers, so that the template can be reused. (L. L. Silver and N. G. Nossal)

Initiation of DNA Chains within Duplex Templates — By analogy with other systems, it seems likely that an early step in T4 DNA synthesis is the

recognition of an origin specific sequence or sequences by one or more proteins which are able to invade the duplex at this site to synthesize primers for the elongation of the leading strand. The sequences and gene products necessary for this initiation step have not been established for T4 in vivo, and the alternative strand displacement rolling circle type replication beginning at a nick has not been rigorously excluded. There is weak circumstantial evidence that mutants in genes 39 and 52 are defective in the initiation of DNA synthesis. The 39, 52, and perhaps 60 gene products have been shown recently by Liu et al. and Stetler et al. to be part of a T4 topoisomerase which catalyzes the ATP-dependent nicking-closing of supercoiled DNA.

We have carried out a series of experiments in vivo to determine whether E. coli RNA polymerase plays a role in the initiation of replication either through formation of a primer which can be elongated by the T4 DNA replication proteins, or by transcriptional activation, i.e., the opening of the DNA helix during transcription is necessary for the binding of other proteins required for the initiation of DNA synthesis. Since E. coli RNA polymerase is required for the synthesis of T4-induced enzymes which are involved in DNA replication, we have temporally separated the synthesis of early enzymes from DNA replication by infecting at a nonpermissive temperature with reversible temperature-sensitive mutants in genes 43 or 45. After a time at high temperature sufficient to make the early proteins, the temperature is lowered to allow DNA synthesis to begin. The addition of the RNA polymerase inhibitor rifampicin at the time of shift to the lower temperature does not change the kinetics of DNA replication. We therefore conclude that concomitant transcription is not required for the initiation of T4 DNA replication. We are currently testing the possibility that a transcript made during the early period at high temperature survives and can be used by the T4 DNA replication proteins.

In vitro, the T4 41 and 61 proteins do not synthesize primers within any duplex DNA, and there is no synthesis other than the extension of existing nicks (and the priming and copying of the displaced strand) with the combination of the 41, 61 and 43, 44-62, 45, and 32 proteins. We find that the synthesis with duplex DNA with these seven enzymes is not increased by the addition of purified E. coli RNA polymerase, T4-modified E. coli RNA polymerase, or the T4 DNA topoisomerase. These negative results do not exclude the possibility that one or more of these proteins is required for initiation, since origin recognition may require additional proteins and/or a topological configuration of T4 DNA not present in our soluble system. We are currently using affinity columns of duplex T4 DNA cellulose to try to isolate factors stimulating duplex DNA synthesis. We are also characterizing the DNA made in extracts of gently lysed infected cells to see if there is preferential synthesis of any part of the T4 genome. DNA synthesis in these extracts is completely dependent on T4 proteins, since it does not occur in extracts of cells infected with 41^- or 45^- mutants, unless they are supplemented with the corresponding purified gene product. Synthesis in a 41^- extract (supplemented with 41 protein) is dependent on the addition of exogenous DNA and appears to show some preference for T4 DNA. (L. L. Silver, M. Venkatesan, and N. G. Nossal)

A New T4 Deoxyribonuclease — In the course of the studies described above, characterizing DNA molecules made in vitro in crude extracts of infected cells, we have discovered what appears to be a T4 deoxyribonuclease which produces products giving discrete bands on agarose gel electrophoresis. This "nuclease" activity requires a protein made late in the infection cycle since it is not present in uninfected cells or after infection with a mutant in genes 33 and 55, which is defective in all late protein synthesis. The activity is present after infection with wild type phage, as well as with mutants in several other genes whose phenotypes suggest they may control a nuclease (46, 47, 49, den A, den B, rII). We are now testing other mutants in late T4 genes to try to identify the gene(s) responsible for this activity.

The "nuclease" degrades T7 and λ DNA, but has little or no activity on T4 DNA. The pattern of product bands is too complex for a simple restriction enzyme. We are currently purifying this activity to determine whether it is a mixture of sequence-specific restriction nucleases, or is a single protein whose degradation is determined by some other structural feature of the DNA. (N. G. Nossal)

Collaborative Projects

(1) The Effect of Template Secondary Structure on DNA Synthesis by T4 Proteins — Englund and his collaborators have used alkaline agarose electrophoresis of the products of the copying of primed single-stranded templates to show that both vaccinia virus DNA polymerase and T4 DNA polymerase pause at barriers created by internal hairpins in a single-stranded template. We have now shown that the addition of the 44-62 and 45 proteins enables the T4 polymerase to pass through these barriers more efficiently. We are currently correlating the turnover of dNTP (DNA-dependent conversion of dNTP to dNMP) by the polymerase alone or with 44-62 and 45 with its position along the template to test the hypothesis that most of the turnover by the proofreading exonuclease of the polymerase occurs when the polymerase is retarded by secondary features in the template. (N. G. Nossal with Annette Roth and Paul Englund, Johns Hopkins University, Baltimore, Maryland)

(2) Sequence of the T4 45 Protein — The amino acid sequence of this protein is being determined by a combination of direct sequencing of the purified protein, and DNA sequencing of a cloned T4 DNA fragment containing the promoter and structural regions of gene 45. (N. G. Nossal with Kenneth Williams, Elenor Spicer, and William Konigsberg, Yale University, New Haven, Connecticut)

Publications:

McMacken, R., and Nossal, N. G.: Procaryotic replication systems. In Alberts, B., and Fox, C. F. (Eds.): Mechanistic Studies of DNA Replication and Genetic Recombination, ICN-UCLA Symposia on Molecular and Cellular Biology. New York, Academic Press, 1980, Vol. 19, in press.

Nossal, N. G.: DNA replication with bacteriophage T4 proteins: purification of the proteins encoded by T4 genes 41, 45, 44, and 62 using a complementation assay. J. Biol. Chem. 254: 6026-6031, 1979.

Nossal, N. G.: RNA priming of DNA replication by bacteriophage T4 proteins. J. Biol. Chem. 255: 2176-2182, 1980.

Nossal, N. G., and Peterlin, B. M.: DNA replication by bacteriophage T4 proteins: the T4, 43, 32, 44-62, and 45 proteins are required for strand displacement synthesis at nicks in duplex DNA. J. Biol. Chem. 254: 6032-6037, 1979.

Silver, L. L., Venkatesan, M., and Nossal, N. G.: RNA priming and DNA synthesis by bacteriophage T4 proteins. In Alberts, B., and Fox, C. F. (Eds.): Mechanistic Studies of DNA Replication and Genetic Recombination, ICN-UCLA Symposia on Molecular and Cellular Biology. New York Academic Press, 1980, Vol. 19, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 24,360-06 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Action of Thrombin on Fibrinogen and Study of Related Blood Clotting Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Albert J. Osbahr, Ph.D. Research Biochemist LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biophysical Biochemistry

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Thrombin enzymatically released two fibrinopeptides from citraconylated canine fibrinogen. The rate of removal of modified fibrinopeptide-A was increased 40% over the rate of release of peptide-A from native canine fibrinogen. The modification of 80 or more lysine groups inhibited peptide release which was attributed to a conformational change in the structure of fibrinogen. Edman analysis verified the presence of a modified lysine in fibrinopeptide-A. The peptide release from human fibrinogen, a species lacking lysine in peptide-A, was not altered by modification. On the other hand, the rate of release of bovine fibrinopeptide-B by thrombin was increased by citraconylation of the lysine residue in the peptide. The presence of a carboxylic acid residue in the 6th position from the arginyl-glycyl bond cleaved by thrombin influences the rate of release of the peptides. Citraconylation of fibrinogen can be reversed without disturbing the periodicity obtained during polymer formation. Radioactive glycine ethyl ester incorporated glycine residues into fibrinogen, revealing that 4 of the 5 carboxylic acid residues in fibrinopeptide-A were modified during methylation. The glutamic acid residue in the 6th position was implicated in the reduction of thrombin activity toward methylated fibrinogen.

Project Description:

Objectives:

This study involves a structural analysis of the blood coagulation process and the mechanism of the physiological action of thrombin on fibrinogen. The biological activity of the fibrinopeptides and their role in hemostasis is also under investigation. The structure-function relationships of the blood coagulation proteins are of particular interest to increase our knowledge and understanding of the mechanisms involved in these vital blood clotting processes.

Chemical modification of the functional groups of the amino acids that dictate protein structure should advance our knowledge and aid us in our endeavor to establish the mode of action of these very significant biological reactions. Our present interest involves amino acids that can generate positive or negative charges in the protein structure such as the ε-amino group of lysine and the carboxyl groups of aspartic and glutamic acid. The modification of these residues with specific modifying agents may advance our understanding of the important mechanisms involved in hemostasis, such as the conversion of fibrinogen to fibrin by thrombin, the aggregation of platelets, and the polymerization of monomeric fibrin into the fibrin clot.

Methods Employed:

The proteins and peptides of the coagulation process were analyzed for composition and structure by the methods of column chromatography, spectrophotometric analysis, and high voltage electrophoresis. Ultracentrifugation, thin-layer chromatography, paper and column chromatography, and disc gel electrophoresis were used to evaluate the purity of the protein and peptide systems. Proteolytic enzymes and chemical methods were employed for structure determinations.

Chemical group specific agents were used to alter the structure of designated amino acid residues. The degree of alteration of the systems was determined by such methods as amino acid analysis, dansylation, ninhydrin, radioactive incorporation, and specific amino acid procedures.

Major Findings and Proposed Course of Study:

Thrombin Digestion of Citraconylated Canine Fibrinogen. Citraconic anhydride was employed to modify the lysine residues of canine fibrinogen. The extent of modification was followed by the dansyl method and amino acid analysis. Incubation of the citraconylated canine fibrinogen with thrombin was carried out at 37° C and at no point did aggregates of fibrin polymers appear. The reaction mixture was separated and the fibrinopeptides isolated and purified by column chromatography. The rate of removal of modified fibrinopeptide-A was increased 40% over the rate obtained for the action of thrombin on native canine fibrinogen in a comparative analysis. It was determined that the rate of cleavage of modified fibrinopeptide-A was regulated by the degree of citraconylation of the fibrinogen. At a level of 80 or more lysine residues

modified, inhibition of peptide release occurred. This inhibition was attributed to a conformational change in the structure of citraconylated fibrinogen induced by the high incorporation of negative charge into the molecule. If by the presence of this high negative environment, the site of thrombin action was masked, then the efficiency of the hydrolytic cleavage would have been reduced. The application of the Edman method on the citraconylated peptide-A verified the presence of a modified lysine group. The action of trypsin on the modified peptide failed to hydrolyze at the internal lysine site, thereby further confirming the presence of a covalent linkage at the ε-amino group of lysine.

Citraconylation of a Species of Fibrinogen Lacking Lysine in Fibrinopeptide-A. Human fibrinogen does not contain a lysine residue in its fibrinopeptide-A and would, therefore, make a good model to test the involvement of the citraconylated lysine, with the additional negative charge it imposes on the peptide, in the enhancement of the rate of release of canine fibrinopeptide-A from fibrinogen. The citraconylation of human fibrinogen was achieved, and a product which had 40 lysine groups modified was reacted with thrombin. The rate of release of fibrinopeptide-A from the modified human fibrinogen was not significantly altered by modification. Therefore, although citraconylation of lysine residues had occurred elsewhere in the fibrinogen chains, it had neither accelerated nor inhibited the action of thrombin at the site of cleavage of the fibrinopeptide. This comparative study, therefore, confirmed our proposed mechanism that the increased activity of thrombin on citraconylated canine fibrinogen was a result of the incorporation of negative charge into the fibrinopeptide-A, at the lysine residue, resulting in a faster removal rate of the peptide by the hydrolytic cleavage of thrombin.

Structure-Function Relationship of Charged Residues in the Fibrinopeptide Cleavage. It was determined that fibrinopeptide-A of canine and bovine fibrinogens are initially released at a faster rate than the respective B peptides by thrombin. The structure of each fibrinopeptide-A contained a carboxylic acid residue in the sixth position from the bond enzymatically cleaved by thrombin. Inspection of the structure of bovine fibrinopeptide-B reveals that it is lacking a carboxylic acid residue at this location in the peptide. However, the structure does contain a lysine group in the seventh position from the thrombin cleavage site. Bovine fibrinogen was citraconylated and after isolation and purification was reacted with 50 units of thrombin. The fibrinopeptides were separated and analyzed. Fibrinopeptide-B was found to have been citraconylated at its lysine residue, which generated a negative charge at this position in the peptide. The rate of release of peptide-B from bovine fibrinogen was studied and was determined to have been increased over and above the rate of cleavage of unmodified fibrinopeptide-B from unmodified fibrinogen. The presence of negative charge in the approximate vicinity of six residues removed from the arginyl-glycyl bond hydrolyzed by thrombin was, therefore, influential in the enzymatic cleavage of an otherwise slower releasing fibrinopeptide from fibrinogen.

Removal of the Citraconyl Groups from Modified Fibrinogen. Citraconylated bovine fibrinogen was incubated at 37° C in 0.2 M pyridine-acetate buffer at pH 4.0 for 20 hours during which time the citraconyl groups were removed from the lysine residues. Dansylation confirmed the regeneration of the free ε-amino groups of lysine. The regenerated bovine fibrinogen was subjected to the action of thrombin which resulted in the formation of a fibrin clot. Purification of the clot liquor revealed the presence of two fibrinopeptides. A profile of the rate of release of these peptides from the regenerated fibrinogen was identical with the normal rate of release from native bovine fibrinogen by thrombin. The fibrin clot produced by thrombin from the regenerated fibrinogen was subjected to an electron microscopic study, and the electron micrographs obtained resembled very closely the unmodified fibrinogen-fibrin polyers which reflected a highly ordered structure possessing periodicity. This reversibility was evidence that the citraconylation of lysine residues in the fibrinogen had not produced an irreversible conformational change in the protein which, upon regeneration of the free ε-amino groups of lysine, would have interfered with the formation of the expected polymer.

Incorporation of Radioactive Glycine into Fibrinogen. Bovine fibrinogen was methylated with dimethyl sulfate, and the degree of methylation was determined by the incorporation of radioactive glycine using glycine-1-^{14}C ethyl ester. The presence of free carboxyl groups was estimated before and after the methylation procedure. The action of thrombin on a fibrinogen sample that had approximately 100 carboxyl groups methylated released the methylated fibrinopeptides at a drastically reduced rate. The rate of removal of methylated fibrinopeptide-A from methylated fibrinogen was decreased 70% in comparison with unmodified fibrinogen. Analysis of methylated fibrinopeptide-A revealed that four of its five carboxyl groups were methylated. Structural analysis, after proteolytic cleavage of the peptide by chymotrypsin, identified the unmethylated carboxyl group which incorporated radioactive glycine, as residing penultimate to the phenylalanine residue. Further analysis determined that the glutamic acid residue six positions removed from the enzymatically susceptible arginyl-glycyl bond, which was proteolytically cleaved by thrombin in the clotting of fibrinogen, was methylated. From previous structure-function experimentation involving the positioning of negative charge in the fibrinopeptides, we can significantly implicate the drastic reduction of thrombin activity toward fibrinogen as proceeding from the reduction of negative charge of this glutamic acid residue by methylation.

Significance to Biomedical Research and the Program of the Institute:

Modification of the blood clotting proteins may well serve as a very informative model for the future investigation of the thrombin-induced polymerization of fibrinogen, as well as, a possible diagnostic application in our ultimate control of the mechanism of clot formation in the absence of needed hemostasis. The understanding of thrombus formation in blood vessels is of great importance to our eventual control of undesirable hemostasis which can be quite destructive to life.

Our studies indicate that there is a relationship between the peptides from the blood clotting protein fibrinogen and the physiological processes in blood circulation. It is hoped that investigation of the structure-function analysis of the blood clotting proteins may result in a deeper understanding of the mechanisms associated with hemostasis, vasoconstriction, and blood coagulation. The greater our knowledge concerning the action of these peptides and proteins, the easier our task could be in sorting out and defining the mechanisms related to clotting diseases.

Proposed Course:

A continuation of the modification of fibrinogen will be made to more fully characterize the mechanisms involved in blood coagulation, hemostasis, and vasoconstriction. Studies on the rate of release of the fibrinopeptides from fibrinogen which has been both citraconylated and methylated will also be undertaken to further define the mechanism of action of thrombin. In addition, the citraconylated and methylated fibrinogens will be characterized as to solubility, polymerization phase, conformational effects of modification, and proteolytic degradation by plasmin.

Further efforts will be pursued to more fully characterize the effects of methylation of fibrinogen on the stabilization of the clot formation. The effect of citraconylation of lysine residues on the cross-linking reactions of fibrinogen will be investigated in an effort to determine what alterations this modification could have upon the action of the enzymes Factor XIII and transglutaminase with fibrinogen. Preliminary work on the clotting mechanism in shark's blood will be continued to establish the physiological activity and structure of the fibrinopeptides of this species of fibrinogen in comparison to the previous fibrinogens studied which should advance our understanding of the structure-function relationships in the hemostatic process.

Studies on the modification of fibrinogen will be extended to other functional groups to obtain a greater insight into the relationship between structure and biological activity. Attempts will also be made to establish the role of muscle receptor sites in the mediation of the fibrinopeptide participation in the mechanism of hemostasis via the process of vasoconstriction.

Publications:

Osbahr, A. J.: Fibrinogen-induced polymerization via the process of methylation. Thromb. Haemostasis 42: 1398-1410, 1980.

Osbahr, A. J.: The polymerization of fibrinogen under the influence of diazomethane modification. Biomaterials 1: 100-102, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,390-07 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Hormones and Receptors: Insulin and Insulin-Like Growth Factors (Somatomedins)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Matthew M. Rechler, M.D.	Medical Director, USPHS	LBP NIAMDD
OTHERS:	Alfred B. Knight, M.D.	Intergovernmental Personnel Act Appointee	LBP NIAMDD
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	Joyce A. Romanus	Biologist	LBP NIAMDD

COOPERATING UNITS (if any) C.R. Kahn, G.S. King, and J.M. Podskalny (DB, NIAMDD); A.C. Moses, S.P. Nissley, P.A. Short, and R.M. White (MB, NCI); E.R. Froesch and J. Zapf (Kantonspittal, Zurich, Switzerland); R.E. Humbel (Univ. Zurich, Switzerland); T.D. Gelehrter and J. Heaton (Univ. Michigan); A.M. Rosenberg (Univ. Manitoba, Canada)

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biochemistry of Cell Regulation

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.8	5.5	0.3

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Fibroblasts established in culture from skin biopsies have cell surface receptors for the polypeptide hormones, insulin and somatomedin. We have identified the first selective genetic defect of insulin receptors in fibroblasts cultured from a patient with the syndrome of leprechaunism, characterized by physical abnormalities, intrauterine growth retardation, and clinical insulin resistance. The functional consequences of this receptor defect are under investigation. In our studies of the insulin-like growth factor (somatomedin) multiplication stimulating activity (MSA), a polypeptide synthesized by a cloned line of rat liver cells (BRL 3A), progress has been made in the following areas: (i) purification of MSA to homogeneity and determination of its amino acid composition; (ii) development of a radioimmunoassay for MSA; (iii) demonstration that receptors for MSA and the human somatomedins IGF I and IGF II are remarkably diverse, although each receptor recognizes the 3 peptides; (iv) demonstration that insulin and MSA utilize insulin and MSA receptors, respectively, to mediate the same biological responses in rat hepatoma cells; (v) direct demonstration that insulin and MSA both stimulate glucose oxidation in rat adipocytes via the insulin receptor; (vi) demonstration that MSA is the major somatomedin in fetal rat serum, and that MSA levels fall shortly after birth; (vii) characterization of growth hormone-dependent binding proteins for somatomedins in rat and human serum.

Project Description:

I. Insulin Receptors in Genetic Disorders of Insulin Resistance

Fibroblast cultures established from skin biopsies and propagated for many cell generations in vitro provide a useful cell type to study the genetic endowment of patient's cells free from in vivo physiological influences. We have been interested in genetic syndromes of target-organ resistance to the polypeptide hormones, insulin and somatomedins, and in the cellular mechanisms of hormone resistance.

We first demonstrated that normal human fibroblasts in culture possess separate, specific cell surface receptors for insulin and somatomedins. We have examined insulin receptors in two genetic syndromes associated with insulin resistance: ataxia telangiectasia and leprechaunism. In ataxia telangiectasia, circulating antibodies to insulin receptors decrease insulin binding to monocytes freshly isolated from the patient. Insulin binding to fibroblasts cultured from these patients was normal, however, indicating that the insulin receptor itself was intrinsically normal.

By contrast, we have identified the first primary defect of insulin receptors in a patient with the rare genetic syndrome of leprechaunism. Leprechaunism is characterized by low birth weight, abnormal facial features, insulin resistance, and early death. Binding of ^{125}I-labeled insulin to high affinity insulin receptors was virtually absent in fibroblasts cultured from the leprechaun patient, whereas binding of a chemically unrelated polypeptide, EGF, was normal. Studies are in progress to identify the functional consequence of this receptor defect, including glucose metabolism, amino acid transport, and DNA synthesis. The genetic lesion in this leprechaun patient's fibroblasts promises to provide a unique opportunity to understand how a single biological signal is transmitted and expressed as multiple biological effects.

II. Multiplication Stimulating Activity

Multiplication stimulating activity (MSA) has been purified from media conditioned by a cloned line of rat liver cells in culture. An 8,700 molecular weight form of MSA has been purified to homogeneity and its amino acid composition determined. Glycine is the COOH-terminal amino acid; the NH_2-terminus is blocked. Specific competitive binding assays for MSA have been developed utilizing ^{125}I-labeled MSA binding to cell surface receptors, serum carrier proteins, and rabbit antibodies to MSA. MSA closely resembles the family of polypeptide hormones in human plasma known as somatomedins or insulin-like growth factors in its biological, chemical, and binding properties. Our main interests include: (a) determination of the amino acid sequence of MSA; (b) study of MSA biosynthesis in cell culture and cell-free systems; (c) evaluation of MSA as a specific fetal growth factor; (d) characterization of receptors for MSA, other somatomedins, and insulin, in order to understand the relationships among these receptors and how they are linked to specific

biological functions; and (e) purification, chemical characterization, and development of assays for somatomedin binding proteins, in order to understand their regulation and biological role.

MSA has been compared with insulin-like growth factors IGF I and IGF II, the two human somatomedins whose amino sequences have been completely defined, in multiple receptor assay systems. The three labeled and unlabeled peptides were examined in competitive binding assays using receptors from fibroblasts and hepatocytes of different species. The three peptides cross-reacted in each experimental system, with potencies of 10-100%, further supporting inclusion of MSA in the family of insulin-like growth factors. Subtle differences in binding of the three radiolabeled peptides were appreciated, indicating a rather remarkable diversity of IGF receptors. Whether the IGF receptors of different specificity mediate different biological functions is presently unknown.

Many tissues possess receptors for both insulin and MSA. Although these receptors have distinct specificities, MSA cross-reacts with insulin receptors and insulin cross-reacts with some MSA receptors, presumably because of chemical similarities in the polypeptides. In many tissues, insulin and MSA elicit the same biological responses, raising the question whether these effects are mediated by insulin receptors or MSA receptors. In HTC rat hepatoma cells, insulin and MSA induce the enzyme tyrosine aminotransferase and stimulate amino acid transport. The biological dose-response curves and the specificity of insulin and MSA receptors in HTC cells suggest that insulin acts exclusively via the insulin receptor, whereas MSA may act via the MSA receptor, the insulin receptor, or both.

A more direct analysis has been made of the receptor mechanisms by which insulin and MSA stimulate glucose oxidation in adipocytes and DNA synthesis in cultured human fibroblasts. These studies used Fab fragments prepared from antibodies to insulin receptors (obtained from the serum of a patient with a rare form of insulin-resistant diabetes mellitus) to selectively block insulin receptors (without affecting MSA receptors) and to assess the effect of this blockade on the biological effects of insulin and MSA. These studies demonstrated that both insulin and MSA stimulate glucose oxidation in isolated rat adipocytes via insulin receptors. By contrast, the stimulation by insulin and MSA of DNA synthesis in human fibroblasts in culture is not mediated by insulin receptors, but rather by receptors for MSA or other growth factors.

A possible role for MSA as an intrauterine growth factor has been suggested by two separate experimental approaches: (i) explants of term fetal rat liver studied in short-term organ culture synthesize significant quantities of MSA and (ii) serum from rat fetuses at term contain 20 to 100 times more MSA than the corresponding maternal sera. The level of MSA decreases to adult levels by 10-20 days after gestation. Immunoreactive MSA could account for all of the somatomedin activity in fetal rat serum, but probably constitutes 5-10% of the total somatomedin activity in serum from young and adult rats.

Rat and human plasma contain specific carrier proteins for somatomedins (identified by their ability to bind ^{125}I-MSA). In both rat and human serum, the concentration and molecular size of the somatomedin carrier proteins appear to be determined by the growth hormone status of the animal or patient. Hypophysectomized rats and growth hormone-deficient patients have low levels of carrier protein (≅ albumin-size). Following in vivo administration of growth hormone, the concentration of carrier protein increases dramatically to normal levels, and, as in normals, the binding protein is predominantly globulin-size.

Publications:

Heaton, J. H., Schilling, E. E., Gelehrter, T. D., Rechler, M. M., Spencer, C. J., and Nissley, S. P.: Induction of tyrosine aminotransferase and amino acid transport in rat hepatoma cells by insulin and the insulin-like growth factor, MSA: medication by insulin and MSA receptors. Biochim. Biophys. Acta, in press.

King, G. L., Kahn, C. R., Rechler, M. M., and Nissley, S. P.: Direct demonstration of separate receptors for growth and metabolic activities of insulin and multiplication-stimulating activity (an insulin-like growth factor) using antibodies to the insulin receptor. J. Clin. Invest. 66: 130-140, 1980.

Moses, A. C., Nissley, S. P., Rechler, M. M., Short, P. A., and Podskalny, J. M.: The purification and characterization of multiplication stimulating activity (MSA) from media conditioned by a rat liver cell line. In Giordano, G., Van Wyk, J. J., and Minuto, F. (Eds.): Somatomedins and Growth, Proceedings of the Serono Symposia, London, Academic Press, 1979, Vol. 23, pp. 45-59.

Moses, A. C., Nissley, S. P., Short, P. A., and Rechler, M. M.: Immunological cross-reactivity of multiplication stimulating activity polypeptides. Eur. J. Biochem. 103: 401-408, 1980.

Moses, A. C., Nissley, S. P., Short, P. A., Rechler, M. M., and Podskalny, J. M.: Purification and characterization of multiplication-stimulating activity. Insulin-like growth factors purified from rat-liver-cell-conditioned medium. Eur. J. Biochem. 103: 387-400, 1980.

Muggeo, M., Kahn, C. R., Bar, R. S., Rechler, M. M., Flier, J. S., and Roth, J.: The underlying insulin receptor in patients with antireceptor autoantibodies: demonstration of normal binding and immunologic properties. J. Clin. Endocrinol. Metab. 49: 110-119, 1979.

Nissley, S. P., and Rechler, M. M.: Multiplication-stimulating activity from the BRL-3A rat liver cell line: relation to human somatomedins. Proceedings of the 6th International Congress of Endocrinology, Melbourne, Australia, in press.

Nissley, S. P., Rechler, M. M., Moses, A. C., Eisen, H. J., Higa, O. Z., Short, P. A., Fennoy, I., Bruni, C. B., and White, R. M.: Evidence that

multiplication stimulating activity, purified from the BRL-3A rat liver cell line, is found in rat serum and fetal liver organ cultures. In Hormones and Cell Culture, Cold Spring Harbor Conferences on Cell Proliferation. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press, 1979, Vol. 6, pp. 79-94.

Rechler, M. M.: Receptors for somatomedins and related peptides. In Waldhäusl, W. K. (Ed.): Diabetes 1979, International Congress Series No. 500. The Netherlands, Elsevier North-Holland, 1980, pp. 226-271.

Rechler, M. M., Eisen, H. J., Higa, O. Z., Nissley, S. P., Moses, A. C., Schilling, E. E., Fennoy, I., Bruni, C. B., Phillips, L. S., and Baird, K. L.: Characterization of a somatomedin (insulin-like growth factor) synthesized by fetal rat liver organ cultures. J. Biol. Chem. 254: 7942-7950, 1979.

Rechler, M. M., Nissley, S. P., King, G. L., Moses, A. C., Schilling-Van Obberghen, E., Romanus, J. A., Knight, A. B., Short, P. A., and White, R. M.: Multiplication stimulating activity (MSA) from the BRL-3A rat liver cell line: relation to human somatomedins and insulin. J. Supramol. Struct., in press.

Rechler, M. M., Schilling, E. E., King, G. S., Fraioli, F., Rosenberg, A. M., Higa, O. Z., Podskalny, J. M., Grunfeld, C., Nissley, S. P., and Kahn, C. R.: Receptors for insulin and insulin-like growth factors in disease. In Pepeu, G., Kuhar, M. J., and Enna, S. J. (Eds.): Receptors for Neurotransmitters and Peptide Hormones. New York, Raven Press, 1980, pp. 489-497.

Rechler, M. M., Zapf, J., Nissley, S. P., Froesch, E. R., Moses, A. C., Podskalny, J. M., Schilling, E. E., and Humbel, R. E.: Interactions of insulin-like growth factors I and II and multiplication-stimulating activity with receptors and serum carrier proteins. Endocrinology, in press.

Rosenberg, A. M., Haworth, J. C., Degroot, G. W., Trevenen, C. L., and Rechler, M. M.: A case of leprechaunism with severe hyperinsulinemia. Am. J. Dis. Child. 134: 170-175, 1980.

Schilling, E. E., Rechler, M. M., Grunfeld, C., and Rosenberg, A. M.: Primary defect of insulin receptors in skin fibroblasts cultured from an infant with leprechaunism and insulin resistance. Proc. Natl. Acad. Sci. U.S.A. 76: 5877-5881, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,590-09 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure and Interactions of Biologically Important Macromolecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Harry A. Saroff, Ph.D. Scientist Emeritus and Guest Worker LBP NIAMDD

COOPERATING UNITS (if any)
Clinical Endocrinology Branch, NIAMDD; James C. Osborne, Ph.D., Molecular Disease Branch, NHLBI; Elvin A. Kabat, Ph.D., Columbia University College of Physicians and Surgeons, New York, New York (and Consultant to NIH at present)

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cooperative binding systems involving aggregation, redistribution, subunit interactions, and ligand interactions are being formulated and studied in hemoglobin and other proteins related to hormone action.

Slow degradation of hemoglobin, thyroglobulin, immunoglobulins, and albumin is under investigation in order to define and explain the phenomenon.

Monte Carlo analyses are done to yield probabilities for uniqueness and homology of amino acid sequences.

PHS-6040

Project Description:

Objectives:

The main objective of this program is an elucidation of the structure of naturally occurring macromolecules based on the methods of physical chemistry. Macromolecules are studied primarily in solution, and the emphasis is on the accumulation of data on the state of the molecule with respect to its environment. Gross features of structures as well as fine detail may be observed by studies.

Methods:

In our studies aimed at finer details of protein structure, the methods used involve the measurements of the binding of small molecules or ions to specific sites on the protein molecules. Depending on our needs, we employ ultracentrifugal, electrophoretic, light scattering, and other spectral techniques.

Major Findings and Proposed Course of Studies:

I. Binding Studies and Protein Interactions. Model systems are being developed and studied for explaining in a reasonable manner experimental binding data. A large body of biological data appears to fall in the category of cooperative equilibria and our efforts are directed to this area.

II. Slow (Spontaneous) Degradation of Proteins. Many examples of nonenzymatic cleavage of peptide bonds have been reported in the literature. Some of the conditions are unusually mild involving only hydrogen and hydroxyl ions. The intrinsic internal environment of protein molecules affords electronic configurations which would lend themselves to autocatalytic enzymatic action given transient high potential regions. It is reasonable to assume that the evolutionary process would, in some instances, employ this intrinsic property of the protein molecule.

In our studies on the spontaneous degradation of proteins we are investigating the aggregation reactions of proteins in a search for a covalent component of this reaction related to spontaneous degradation. Progress in these studies so far has been equivocal.

III. Statistical Studies on Protein Sequences. In our studies on amino acid sequences of proteins we have introduced the concept of uniqueness and a method for its quantitative evaluation. We have shown that proteins have a significant probability of containing tripeptides which enable the identification of both the region of the molecule from which the tripeptide originated as well as the molecule itself against the background of all the other proteins within a given species.

Significance to Biomedical Research and the Program of the Institute:

Our emphasis is on the relationship between chemical properties and the

structure of macromolecules. Once these properties are properly related, the attack on the relationship between structure and function can proceed on a solid basis. The proteins and macromolecules studied are either enzymes or those of interest in biological systems. Our systems are highly controlled with reference to the conditions of the protein environment and the biomedical significance of our findings derive from extrapolation to the biological system. Studies on the binding properties of protein systems related to certain hormones provide insight to possible modes of cooperative effects in hormone action.

Publications:

Saroff, H. A.: Book review of Cooperative Equilibria in Physical Biochemistry by D. Poland. Q. Rev. Biol. 54: 324, 1979.

Orr, H. T., Robb, R. J., Parham, P., Ploegh, H. L., Mann, D. L., Bilofsky, H., Saroff, H. A., Wu, T. T., Kabat, E. A., and Strominger, J. L.: Homology of amino acid sequences in human histocompability antigens (HLA-A and HLA-B) with those in immunoglobulins. Scand. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 24,640-09 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

(I) Reactions of Hemoglobin and (II) Metabolism of Antimony

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard B. Simpson, Ph.D. Research Chemist LBP NIAMDD

COOPERATING UNITS (if any)

Dr. Jonathan D. Berman, Laboratory of Clinical Investigation, NIAID, and Dr. Hideo Kon, Laboratory of Chemical Physics, NIAMDD

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The rates of oxidation of the α and β subunits of hemoglobin by ferricyanide were found to be about equal.

Reaction of hemoglobin with hydroxylamine between pH 7 and 9 produces two unidentified low-spin triplets in the electron spin resonance spectrum. Although these triplets do not correspond in position to those produced by reaction of several simple amines with ferric hemoglobin, one does correspond in position to a triplet produced by reaction of heme with a thiol and imidazole, suggesting that the ferric ion position ordinarily occupied by a water molecule or hydroxyl in methemoglobin is replaced by a cysteine sulfhydryl when hydroxylamine reacts with hemoglobin.

After venous injection of about 15 mg pentavalent antimony per liter, no trivalent antimony above the limits of polarographic detection (approximately 1 mg per liter) could be found, suggesting that conversion to the more toxic trivalent ion does not occur during the killing of the leishmaniasis trypanosome.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To obtain some insight into the mechanism of reactions of hemoglobin, a study is being made of the kinetics and products of reactions of various agents that oxidize the heme.

In collaboration with Dr. Jonathan D. Berman (LCI, NIAID), an investigation is being conducted of the mechanism of action of antimony in killing the leishmaniasis trypanosome.

Methods Employed:

Oxyhemoglobin was prepared from blood by standard methods. The spectral change on oxidation of oxyhemoglobin was measured in the Durrum stopped-flow spectrophotometer. The rate of oxidation by stoichiometric amounts of copper (which oxidizes only the β hemes rapidly) was measured at various wavelengths to determine the isosbestic points for oxidation of the β hemes. After oxidation by copper of the β hemes, the rate of oxidation of the remaining ferrous α subunits by ferricyanide was measured to determine the isosbestic points for the α hemes. Then the oxidation of (entirely ferrous) oxyhemoglobin by ferricyanide was measured at a pair of isosbestic points to compare the rates of oxidation of the α and β subunits by this reagent.

Electron spin resonance spectra of hemoglobin solutions frozen soon after completion of reaction were run in the Varian instrument operated by Dr. Hideo Kon (LCP, NIAMDD).

Patients were given venous injections of pentavalent antimony (Pentastam). At various times after injection, blood was drawn, red cells removed by centrifugation, protein precipitated by sulfosalicylic acid, and the supernatant analyzed polarographically in 1 M hydrochloric acid, a medium in which trivalent but not pentavalent antimony is reduced. Controls consisted of addition of antimony trichloride to the red cell supernatant, since literature reports showed that antimony is not absorbed by erythrocytes.

Major Findings:

From measurements at the isosbestic points for oxidation of the α and β hemes, it was shown that the rates of oxidation of the α and β hemes by ferricyanide are about equal.

Reaction of oxyhemoglobin with hydroxylamine between pH 7 and 8 produces, in addition to the nitric oxide-hemoglobin signal, two unidentified low-spin triplets in the electron spin resonance spectra. To investigate a hypothesis that these peaks are due to complexation with unreacted hydroxylamine, the spectra of hemoglobin oxidized by ferricyanide and then treated with ammonia, methoxyamine, or Tris were measured, and each showed a triplet, but not

corresponding in field strength to either of the triplets produced by hydroxylamine. Another hypothetically possible product of the hydroxylamine reaction, diimide, showed no low-spin peaks on reaction with ferric hemoglobin. One of the hydroxylamine triplets was, however, similar in field strength to the peaks reported for treatment of heme with a thiol and imidazole, suggesting that the ferric ion position ordinarily occupied by a water molecule or hydroxyl in methemoglobin, is replaced by a cysteine sulfhydryl when hydroxylamine reactions with hemoglobin.

During a period of from 2 hours to 3 days after venous injection of about 15 mg pentavalent antimony per liter, no trivalent antimony above the limits of polarographic detection in the plasma (approximately 1 mg per liter) could be found. Although trivalent antimony is known to be more toxic than pentavalent to both humans and leishmaniasis trypanosomes, this finding suggests that conversion to trivalent antimony does not occur in the therapeutic process.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,710-30 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Estimation, Metabolism, and Function of Amines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

			Manyears	
PI:	Herbert Tabor, M.D.	Medical Director, USPHS	1.0	LBP NIAMDD
	and Chief, Laboratory of Biochemical Pharmacology,			LBP NIAMDD
	and Chief, Section on Pharmacology			LBP NIAMDD
	Celia White Tabor, M.D.	Medical Director, USPHS	1.0	LBP NIAMDD
OTHERS:	Stephen M. Boyle, M.D., Ph.D.	Special Expert	0.2	LBP NIAMDD
	Murray S. Cohn, Ph.D.	Staff Fellow and then Guest Worker	0.3	LBP NIAMDD
	George D. Markham, Ph.D.	Staff Fellow	1.0	LBP NIAMDD
	Anil Kumar Tyagi, Ph.D.	Visiting Fellow	0.5	LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	4.0	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are continuing our studies on the biological role of polyamines. Particular attention is being directed towards the selection of Escherichia coli and yeast mutants that are defective in the biosynthesis of these amines, and a study of the resultant phenotypic effects. We are also studying the control of polyamine biosynthesis in the yeast mutants. We are preparing stable, multicopy plasmids which contain the various E. coli genes involved in biosynthesis of the amines, in order to obtain large amounts of the enzymes for study of the protein and cofactors involved.

We have purified and are studying the mechanism of action and the structure of the adenosylmethionine synthetase of E. coli. We are purifying the ornithine decarboxylase of Saccharomyces cerevisiae from a derepressed strain, to study its control.

PHS-6040

Project Description:

Mutations in Spermidine Biosynthesis in Escherichia coli and Saccharomyces cerevisiae.

The polyamines, putrescine and spermidine, are present in E. coli in very high concentration. We are trying to determine the function of these amines in vivo, which is not known despite the multiple in vitro effects which have been described.

We have previously constructed and isolated amine-deficient mutants of E. coli which are completely deficient in putrescine and spermidine as a result of deletions in ornithine decarboxylase, arginine decarboxylase, agmatine ureohydrolase, and adenosylmethionine decarboxylase. We showed that these organisms were still able to grow logarithmically, although slowly compared to wild type cells. These deficient organisms were able to maintain bacteriophages T4, T7, and f2 and to produce bursts of these phages similar to the wild type strains. However, the amine deficiency resulted in a complete inability to produce λ phage, although lysogeny by λ was maintained normally.

By an automated analyzer technique that we had developed earlier, we were able to demonstrate that small amounts of the 5-carbon analog of putrescine, namely, cadaverine, are present in our amine-deficient quadruple mutant strains. We thought that the cadaverine present in the quadruple mutant might account for the growth and phage production in the quadruple mutant. Therefore, we studied the biosynthesis and control of lysine decarboxylase, in order to develop a quintuple mutant unable to synthesize the cadaverine.

We demonstrated that two genes are concerned with lysine decarboxylation: (1) a regulatory gene (cadR) and (2) a structural gene for the lysine decarboxylase (cadA). We developed a screening assay procedure for mass surveys and were able to map cadR at 46 minutes and cadA at 92 minutes. We have incorporated the cadA mutation into the quadruple mutant by standard transduction techniques and have studied the resulting quintuple mutant, which lacks cadaverine as well as putrescine and spermidine. This quintuple mutant showed no significant differences from the quadruple mutant. Thus, while amine deficiency in E. coli produces a moderate decrease in growth rate and is defective in the production of λ bacteriophage, the organisms are still able to grow logarithmically indefinitely.

The defect in λ production appears to be one of our most promising leads in studying the biological role of polyamines. We are investigating the defect in λ development by studying the formation of λ RNA message and λ DNA after induction of lysogenic quintuple mutants and after infecting deficient strains with λ.

Our studies on the E. coli adenosylmethionine decarboxylase in the past have shown that this enzyme contains covalently bound pyruvate. We are investigating the mechanism by which this unusual cofactor is formed and determining

the stoichiometry of the pyruvate to enzyme subunits. We are purifying the enzyme from cells containing the plasmid carrying the speD gene (which we isolated from the Carbon collection) and have been able to develop an affinity column to facilitate the purification procedure. Since this ColE1 plasmid is unstable, we are now attempting to transfer this speD gene to a more stable plasmid such as pBR322, or an R plasmid with high copy number. We hope to be able to find evidence for a proenzyme, to determine the mechanism of biosynthesis of the cofactor pyruvate and to study the mechanism of the reaction in detail.

We have previously identified a plasmid in the Carbon collection which carries the genes for ornithine decarboxylase and another plasmid which carries 3 genes involved in polyamine biosynthesis, namely, arginine decarboxylase, agmatine ureohydrolase, and adenosylmethionine synthetase. Using the new genetic cloning techniques, we are trying to develop new hybrid plasmids with very high copy number in order to facilitate purification and study of the proteins involved in amine biosynthesis.

Studies of the pure adenosylmethionine synthetase with X-ray spectroscopy are being carried out in collaboration with Drs. D. R. Davies and G. L. Gilliland (LMB, NIAMDD). Crystals have been obtained which can be used for studies down to 3A by crystallization in the presence of pyrophosphate and Mg^{2+}.

Studies of the mechanism of the reactions of pure adenosylmethionine synthetase, both in the synthesis of adenosylmethionine and in the tripolyphosphatase reaction, are continuing. Both activities are carried out by the same polypeptide chain. By the use of NMR techniques, we have shown that 2 Mg^{2+} and 1 monovalent ion bind to the active site. The two activities have been dissociated for the first time by the use of the ATP analog AMPPNP. The use of S analogs of ATP has shown that the Mg^{2+} binds to only one of the oxygens on the β-phosphate.

We have previously described yeast mutants completely deficient in polyamines as a result of a mutation in ornithine decarboxylase. In yeast, these strains are completely unable to grow without added amines. We have found that "bypass" mutants which grow up from a lawn of deficient cells are deficient in spermine synthase. Two types of mutations have been found: (1) regulatory mutants and (2) mutants in the structural gene for spermine synthase. Thus, spermine and/or the enzyme that synthesizes spermine from spermidine and decarboxylated adenosylmethionine are very important in the control of putrescine synthesis in yeast. We have also found other mutants which have very high levels of ornithine decarboxylase. We are studying the genetics and biochemistry of these organisms.

Ornithine decarboxylase of eukaryotic cells is of particular interest to many laboratories. It has the shortest half-life of any known mammalian enzyme, and as a result its concentration responds rapidly to situations leading to protein synthesis, i.e., new growth or differentiation. In addition, a very novel type of control results from the presence of an antizyme, reported by Dr. E. S. Cannelakis.

The yeast system is a particularly good system for the study of eukaryote ornithine decarboxylase. We have shown that mutants of yeast which cannot synthesize spermidine or spermine as a result of a defective adenosylmethionine decarboxylase have very high ornithine decarboxylase levels. We are purifying the ornithine decarboxylase from these organisms and are looking for evidence of control mechanisms in yeast, such as an antizyme or modification of this protein.

Publications:

Cohn, M. S., Tabor, C. W., and Tabor, H.: Regulatory mutants affecting ornithine decarboxylase activity in Saccharomyces cerevisiae. J. Bacteriol., in press.

Hafner, E. W., Tabor, C. W., and Tabor, H.: Mutants of Escherichia coli that do not contain 1,4-diaminobutane (putrescine) or spermidine. J. Biol. Chem. 254: 12,419-12,426, 1979.

Markham, G. D., Hafner, E. W., Tabor, C. W., and Tabor, H.: S-Adenosylmethionine synthetase from Escherichia coli. J. Biol. Chem., in press.

Tabor, H., Hafner, E. W., and Tabor, C. W.: Construction of a strain of Escherichia coli unable to synthesize putrescine, spermidine, or cadaverine: characterization of two genes controlling lysine decarboxylase. J. Bacteriol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,940-07 LBP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Killer Double-Stranded RNA Plasmids of Saccharomyces cerevisiae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Reed B. Wickner, M.D.	Senior Surgeon, USPHS	LBP NIAMDD
OTHERS:	Patricia Guerry-Kopecko, Ph.D.	Staff Fellow	LBP NIAMDD
	R. Michael Sheetz, Ph.D.	Staff Fellow	LBP NIAMDD
	Susan Porter Ridley, Ph.D.	Guest Worker (American Cancer Society Postdoctoral Fellow)	LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.6	3.3	0.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The presence of a 1.4 x 10^6 dalton double-stranded RNA in intracellular virus-like particles (the K_1 killer plasmid or killer virus) in Saccharomyces cerevisiae enables cells to secrete a protein toxin and be immune to this toxin. We have defined 29 chromosomal genes (called mak1, . . .) needed to replicate the killer plasmid and 5 regulatory chromosomal genes affecting the killer plasmid (ski genes, KRB1). A second killer plasmid, called K_2, is excluded by the K_1 plasmid. We have found a new plasmid, called [EXL], which is not a killer plasmid but also excludes K_2. Yet another plasmid, called [NEX], prevents this effect of [EXL] on K_2. A chromosomal gene, mkt1, is required for K_2 maintenance if [NEX] is present. A third new yeast plasmid, [HOK], acts as a helper for a replication-defective mutant of the K_1 killer plasmid. [EXL], [NEX], and [HOK] each depend on various MAK genes for their replication.

PHS-6040
(Rev. 10-76)

Project Description:

Background:

Some strains of the eukaryote Saccharomyces cerevisiae secrete a protein toxin which is lethal to other strains. The toxin-secreting strains are called killers and are immune to their own toxin. Two different toxin specificities are known (K_1^+ and K_2^+ phenotypes) with their corresponding immunity (R_1^+ and R_2^+) specificities. K_1 killer strains are killed by the toxin from K_2 killers and vice versa. Ability to secrete an active toxin depends on the presence of a double-stranded (ds) RNA which codes for the toxin and an immunity function. K_1 strains have a 1.25×10^6 dalton ds RNA, called M_1, while K_2 killers carry a smaller 1.0×10^6 dalton ds RNA, M_2. Each ds RNA is found in virus-like particles. Another ds RNA, called L (3.0×10^6 daltons), is found in virtually all S. cerevisiae strains and codes for the coat protein of the virus-like particles in which each ds RNA is encapsulated. The K_1 and K_2 killer traits are inherited as plasmids (denoted [KIL-k_1] and [KIL-k_2]), and the virus-like particles containing L, M_1, or M_2 are not infectious.

We have described two chromosomal genes kex1 and kex2, needed for expressing the killer phenotype, but not needed for resistance or for killer plasmid (M_1 ds RNA) maintenance. The kex2 gene is also necessary for secretion of active α hormone--a pheromone secreted by cells of α mating type which readies a cells for mating--and for meiotic spore formation. The rex1 gene is required for expression of resistance to the toxin, but not for toxin production or plasmid maintenance.

Our studies have also revealed 29 chromosomal genes needed to maintain or replicate the K_1 killer plasmid-virus (mak genes). Mutants in any of these genes result in loss of the M_1 ds RNA, but not of L ds RNA. Thus, M_1 and L must be replicated and maintained by very different enzymes. Several mak genes are also required for optimal growth rate, maintenance of the mitochondrial genome, or meiosis.

We have also described 4 chromosomal genes with regulatory effects on killer plasmid replication (called ski genes because the mutants are "superkillers") and 2 other genes (one chromosomal and one mitochondrial), mutations in any of which suppress mutations in specific mak genes.

We have described two new non-Mendelian genetic elements affecting replication of [KIL-k_2]. One, called [EXL], excludes [KIL-k_2] when both are present in the same cell. The second, called [NEX], prevents [EXL] from excluding [KIL-k_2] in wild type cells, but in cells defective in the chromosomal mkt1 gene, [NEX] itself excludes [KIL-k_2].

New Findings:

We have described a mutant of [KIL-k_1] which is lost (cannot replicate) in most wild type strains. This mutant of M_1 ds RNA, called [KIL-sd_1], can,

however, be maintained and replicated if the cell has a mutation in any of the chromosomal ski genes (sd = ski-dependent). This mutant M_1 ds RNA is defective in an essential replication function coded by the M_1 ds RNA.

While studying this replication-defective killer plasmid, we found that many SKI^+ strains were able to maintain the [KIL-sd_1] plasmid due to the presence of another plasmid which we have named [HOK] for "helper of killer." The [HOK] plasmid is not a ds RNA plasmid, but we do not yet know what its nucleic acid genome is.

The three new plasmids, [EXL], [NEX], and [HOK], each depend on some of the chromosomal genes required by [KIL-k_1] and [KIL-k_2]. Some of these results are shown in the table below.

	PET18	MAK10	MAK3	MAK1	MAK4	MAK16	MKT1
[KIL-k_1]	+	+	+	+	+	+	-
[KIL-k_2]		+				+	+
L ds RNA	-	-	+ (partial)	-	-	-	-
Mitochondrial DNA .	+	-	-	-	-	-	-
[EXL]	+	+	+	+			
[NEX]	-		+		-		-
[HOK]	+	+	-	-	-	-	-

+ = wild type allele required for plasmid maintenance.

Based on their requirements for MAK genes (above), their insensitivity to ethidium and guanidine (which cure the mitochondrial genome and the PSI plasmid, respectively) and their incompatibility relationships, [EXL], [NEX], and [HOK], are distinct from previously described non-Mendelian genetic elements.

2 μ DNA Replication. All S. cerevisiae strains carry a 4 x 10^6 dalton double-stranded circular DNA plasmid called 2 μ DNA. The cellular functions carried by this plasmid are unknown. Recombinant plasmids carrying the 2 μ DNA replicon have been prepared by others but are not stably maintained in yeast. We constructed a stable derivative of such a plasmid carrying the yeast LEU2 gene. This derivative, called pSLe1, is now being used to study the genetics of 2 μ DNA replication.

Molecular Cloning. We have cloned the yeast URA1 gene, the structural gene for dihydroorotate dehydrogenase. This gene is expressed in Escherichia coli complementing the analogous gene, pyrD.

We have also located, on the genetic map of yeast, several of the genes involved in the regulation of acid phosphatase.

Significance to Biomedical Research and the Program of the Institute:

Using the killer system, we have been able to explore, in depth, the

relationships among various plasmids and host (chromosomal) genes. Our surprising discovery of three new non-Mendelian elements involved in the killer system makes it important to consider cytoplasmic genetic elements in future studies of the genetics of eukaryotes.

Publications:

Ouchi, K., Wickner, R. B., Toh-e, A., and Akiyama, H.: Breeding of killer yeasts for saké brewing by cytoduction. J. Ferment. Technol. 57: 483-487, 1979.

Toh-e, A.: Genetic mapping of the pho2, PHO82-pho4, and pho85 loci of yeast. Genetics, in press.

Toh-e, A., Guerry-Kopecko, P., and Wickner, R. B.: A stable plasmid carrying the yeast LEU2 gene and containing only yeast deoxyribonucleic acid. J. Bacteriol. 141: 413-416, 1980.

Toh-e, A., and Wickner, R. B.: A mutant killer plasmid whose replication depends on a chromosomal "superkiller" mutation. Genetics 91: 673-682, 1979.

Toh-e, A., and Wickner, R. B.: "Superkiller" mutations suppress chromosomal mutations affecting double-stranded RNA killer plasmid replication in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 77: 527-530, 1980.

Welsh, J. D., Leibowitz, M. J., and Wickner, R. B.: Virion DNA-independent RNA polymerase from Saccharomyces cerevisiae. Nucleic Acid Res., in press.

Wickner, R. B.: Killer systems in Saccharomyces cerevisiae. In Jones, E. W., Broach, J., and Strathern, J. A. (Eds.): Molecular Biology of Yeast. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press, in press.

Wickner, R. B.: Mapping chromosomal genes of Saccharomyces cerevisiae using an improved genetic mapping method. Genetics 92: 803-821, 1979.

Wickner, R. B.: Plasmids controlling exclusion of the K_2 killer double-stranded RNA plasmid of yeast. Cell, in press.

Wickner, R. B.: The killer double-stranded RNA plasmids of yeast. Plasmid 2: 303-322, 1979.

Wickner, R. B., and Leibowitz, M. J.: mak mutants of yeast: mapping and characterization. J. Bacteriol. 140: 154-160, 1979.

Honors and Awards:

Dr. R. B. Wickner was awarded a fellowship from the Japan Society for the Promotion of Science (to work for 3 months, April 1 to June 30, 1980, at Osaka University with Professor Yasuji Oshima and Dr. Akio Toh-e).

Dr. Wickner - Member of the National Science Foundation Advisory Committee for Physiology and Cellular and Molecular Biology in 1979 and 1980.

Dr. Wickner - U. S. Public Health Service Commendation Award, 1980.

ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

Research in this Laboratory covers a wide range of topics, characterized in part by two themes: a search for the underlying physical basis of molecular and cellular phenomena; and the development of new methods and instruments (often spectroscopic or computer-based) for investigating molecular and cellular structure and dynamics.

During the last year Yoshikami and Hagins have employed new calcium-sensitive microelectrodes to study the diffusion of Ca^{++} into and out of isolated retinas, to study the process by which the retina regulates its extracellular Ca^{++} level, and to detect and measure light-induced Ca^{++} release from rod outer segments during visual excitation. The observed release of Ca^{++} by retinal rods in light conforms to a previously advanced hypothesis that an essential step in visual excitation in vertebrates is the intracellular release of Ca^{++} as a result of photochemical and biochemical reactions of the light-sensitive pigment, rhodopsin.

Charney has extended both his experimental and theoretical investigations on the conformation, dynamics and polyelectrolyte properties of DNA and polynucleotides. Rau and Charney have developed a more complete theory for the polarization of a polyelectrolyte to explain the orientation of DNA in an electric field, and have carried out several new experiments to test the predictions of the theory. Charney and Lee have measured the electric dichroism of low molecular weight DNA in low salt, water-ethanol mixtures, and have confirmed several principal predictions of their theory. The role of charge density in their orientation mechanism has been examined both by varying the charge of the counterions and by using polynucleotides containing different numbers of strands, and has been found to be consistent with theoretical predictions. Using the electric dichroism technique Charney and coworkers have discovered 2 new important structural facts about DNA. Charney and Rau have found that while DNA in solution is in the B form, it is not a straight rod, but is either worm-like or is supercoiled. McGhee, Rau, Charney and Felsenfeld have used the sign and magnitude of the static electric dichroism to postulate models for the relation between the nucleosomes and spacer DNA in multinucleosome particles. In order to examine the arrangement of histones in chromatin, Rau and Charney have developed a new electric dichroism apparatus which extends the useful spectral range of the technique into the region of the peptide absorption bands between 200 and 225 nm.

Hofrichter and Eaton have continued their investigations on sickle cell disease. Ferrone, Hofrichter and Eaton have completed an extensive experimental investigation on the kinetics of sickle cell hemoglobin polymerization, which employed their laser photolysis, light scattering technique. From the results of these studies they have proposed a new mechanism for polymerization. The mechanism postulates 2 pathways for polymer formation. The process is initiated by the homogeneous nucleation of individual polymers in the bulk solution phase, as this group has previously proposed, but is then followed by further nucleation on the surface of pre-existing polymers. This second

NIH 1.1
4-68

pathway for nucleating new polymers can account for the previously unexplained extreme autocatalysis in the kinetic progress curves. The equations describing this model have been formulated in terms of nucleation equilibrium constants that can be evaluated from statistical thermodynamics. Numerical integration of the kinetic differential equations are being carried out to determine the values of the parameters of the model that are compatible with the experimental data. Sunshine, Hofrichter and Eaton have carried out a thermodynamic study to explain quantitatively why sickle deoxyhemoglobin polymerizes, but sickle oxyhemoglobin does not. They have used linear dichroism and solubility measurements to determine the oxygen binding curve of the polymer, and have found that the binding is non-cooperative with an affinity for oxygen which is about 2-1/2 times less than solution hemoglobin in the T quaternary structure. This study has also confirmed the two-phase model for sickle hemoglobin gels in a convincing fashion. To explore the predictions of the "kinetic hypothesis" for the pathophysiology of sickle cell, Hofrichter, Coletta and Eaton are developing the laser photolysis, light scattering technique for studies on single red cells. They will use this method to examine the distribution of intracellular gelation delay times in blood samples from patients with varying degrees of clinical severity, and to test the potential efficacy of gelation inhibitors that may be useful in the treatment of patients with sickle cell disease.

Eaton has compared the coding sequences (exons) of the α and β globin gene with the structure of hemoglobin, and has used the allosteric model to show that the coding sequences correspond to polypeptide sequences which are distinct functional parts of hemoglobin. This correlation supports Gilbert's hypothesis that exons code for individual functional units in proteins and that exon recombination can speed the evolutionary process.

Hofrichter has constructed a new kind of pulse-and-probe transient laser spectrometer which is based on two Nd:YAG lasers and an optical multichannel analyzer. He has measured complete absorption spectra with excellent signal-to-noise in 10^{-8} seconds, and at various times ranging from 10^{-8} to 10^{-2} seconds following the photolytic dissociation of the carbon monoxide complex of hemoglobin. He has tested the instrumentation by confirming previous reports of geminate recombination of carbon monoxide within 10^{-7} sec., followed by spectral sharpening up to 10^{-6} sec. and biomolecular recombination at longer times. These are the initial experiments in an investigation of the dynamics of structural changes in hemoglobin planned by Hofrichter and Eaton.

Kon has extended his application of electron spin resonance methods to biological structures to study nitric oxide (NO) liganded iron(II) porphyrins and spin-labelled red blood cells. He has been able to characterize a well-defined dimeric species of an NO-liganded iron(II) porphyrin, and has proposed from this study that the perturbation of NO-liganded hemoglobin by salicylate results in dimer formation of the NO-heme moieties. This work also suggests that the previously unexplained oxidase-like activity produced in hemoglobin by salicylate is caused by dimer formation, and suggests an entirely new approach for understanding oxygen activation by heme proteins. From a spin label investigation, Kon and Inoue have found that the decay of the magnetic

NIH 1351
4-68

anisotropy introduced in flow-oriented red cells is faster in cells with cross-linked cytoskeletal proteins than in intact cells, indicating an important role of the membrane elasticity in determining the deformability of red cells.

McDiarmid, continuing her investigations of the electronic spectra of small, prototype, organic molecules, has both initiated and employed several different spectroscopic techniques. McDiarmid and Doering (Johns Hopkins University) have formulated and verified new "selection rules" for electron impact spectroscopy. These "rules" applied to trans-1,3-butadiene and several substituted cis- and trans-butadienes have located the long sought $^1A_g \leftarrow {}^1A_g$ valence transition, centered around 7.4 eV; aided in resolving the controversy concerning the Rydberg assignments in butadiene; and suggested that electron spectroscopy may be usable in experimentally determining configuration mixing in excited "states." McDiarmid and Auerbach, using a pulsed, tunable dye laser and several pieces of equipment developed here over the last year, have extended the previously observed 2- and 3-photon resonant multiphoton ionization (MPI) of butadiene to 4-photon resonant MPI, enabling both a new butadiene transition to be observed and the fundamental physics of multiphoton absorption to be investigated. McDiarmid and Auerbach have also developed a fast pulsed valve for use as a source in the cooled molecular beam system they have developed and are now testing for MPI spectroscopy.

Sharpless has used classical and quantum mechanical methods to carry out theoretical studies of the forces determining the 3-dimensional structure and intermolecular interactions of small organic compounds with enzymes and other proteins. In particular, the shapes of the stereoisomers of the visual chromophore retinaldehyde have been predicted from analysis of quantum chemical forces between its constituent atoms. Attempts have been made to use similar analysis to predict the inhibiting effect of flavonoids on the catalytic site of aldose reductase.

Nuclear magnetic resonance continued to represent one of the major techniques used in the Laboratory for attacking problems of molecular structure. Shindo and his collaborators have made further progress on the elucidation of conformational features in the backbone of DNA. Their previous high resolution NMR studies of natural and synthetic polynucleotides in solution had shown conformational variability. By using newly developed methods that permit the study of high resolution NMR in solids, they have now found that a similar situation obtains in fibers of natural DNA in the A form. ^{31}P NMR spectra of fibers of the A form of the synthetic poly(dAdT)·poly(dAdT) show two distinct conformations in the phosphodiester backbone, entirely analogous to the spectra of this polymer in solution. On the other hand, fibers of the same polymer in the A form show only a single uniform conformation.

Khetrapal has used NMR to study the detailed geometry of molecules dissolved in solvents which are themselves liquid crystals in the nematic phase. Under these circumstances the solutes become partially ordered and do not tumble isotropically. Thus the dipolar interactions that are normally averaged to zero for molecules in solution can be measured and permit sensitive determination of proton-proton intramolecular distances. Among mole-

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4-68

cules whose structure has been determined in this way during the last year is N-methylimidazole, where the preferred conformation has been found to have one C-H bond of the methyl group lying in the imidazole plane and pointing away from C_5H. Likewise in phenylsilane the preferred conformation has been found to have one hydrogen of the SiH_3 group in the plane, while the carbon analog toluene has previously been found to have a preferred conformation with all hydrogens out of the ring plane. The conformational preferences are attributed to differences in the charge polarizations in CH and SiH bonds.

Gansow and Shindo have begun to explore the use of ^{57}Fe NMR as a structural probe. Because of the low sensitivity and low natural abundance of this isotope only a few limited studies have previously been carried out. The ^{57}Fe chemical shift range has been mapped out by studying a number of different types of iron compounds, and a study designed to elucidate the relaxation mechanisms of ^{57}Fe in a ferrocene derivative is almost completed.

Becker and collaborators have continued their analysis of the random errors occurring in the measurement of NMR spin-lattice relaxation times. Because such measurements are time-consuming for nuclei of low sensitivity or those present in low concentration, an improvement in the efficiency of the measurement is quite worthwhile. Studies last year established the conditions for optimum measurements when the apparatus provides "ideal" radio frequency pulses. Current investigations are directed toward various instances when the ideal pulse assumption is invalid. One commonly employed method by which the NMR intensity data are cast into a logarithmic form in order to generate a straight-line plot has been found to offer little advantage.

Ziffer and Froussios have continued a study using the mold Rhizopus nigricans to hydrolyze enantioselectively a number of racemic acetates of trans 2-substituted 1-indanols and 1-tetralols. The configurations of the alcohols formed were opposite to that obtained from the unsubstituted esters. The results are consistent with a previously proposed model relating the "effective sizes" of substituents flanking the carbinol carbon with the configuration about the carbinol formed.

Ziffer and Banks have prepared several mono- and di-methyl derivatives of 1,2-indene oxide and 1,2,3,4-tetrahydro-1,2-naphthalene oxide. They found that Li^+ catalyzed the rearrangement of the 1-methyl derivatives to allylic alcohols, while the 2-methyl derivative was unreactive under the reaction conditions. The unsubstituted oxides rearranged to 2-indanone and 2-tetralone under the same conditions.

In continuing their studies on the alkaloids from Amoora rohituka, Weiss and Harmon (University of Houston) have investigated the chemistry of an unprecedented structure involving an ethylene oxide ring in the vicinity of the nitrogen atom. Unfortunately, the lability of the compound has prevented a conclusive structure elucidation. The molecular geometry of palustric acid, a dienic resin acid, was established by Weiss and Silverton (NHLBI) for studies involving the chiral-optical effects of non-planar conjugated dienes.

Gansow and his research group at Michigan State have developed methods for synthesizing a new class of radio tracers called lanthanide cryptates. At NIH he has begun *in vivo* testing of these compounds in collaboration with Kessler (Nuclear Medicine, CC). Initial results indicate that cerium cryptate is rapidly taken up in the brain and other organs of the rabbit. Gamma camera images using ^{141}Ce provided pictures of the outline of the brain and thereafter useful images of the rabbit's vascular system were obtained. Further *in vivo* studies of covalently attached cryptates are in progress.

Levin and colleagues have applied vibrational Raman and infrared spectroscopy to examine both lipid-lipid and lipid-protein interactions in model membrane systems. In particular Lavialle and Levin used Raman spectroscopy to investigate the dependence of lipid-protein associations on lipid bilayer acyl chain saturation in reconstituted liposomes involving the myelin proteolipid (or Folch-Pi) apoprotein. For bilayer recombinants prepared from saturated phospholipids, vibrational parameters describing interchain and intrachain disorder within the hydrophobic portion of the lipid matrix indicated significant bilayer perturbations in contrast to minimal bilayer effects in systems comprised of unsaturated lipid assemblies. The rather different bilayer perturbations induced by the apoprotein were related to the diffusion properties exhibited by saturated and unsaturated lipid matrices.

In characterizing the changes occurring within the interfacial region of the phospholipid molecules forming a membrane bilayer, Bush and Levin have used the acyl chain carbonyl modes to differentiate perturbations and conformational changes occurring within either the 1- or 2-chain of the lipid molecule. In addition, Bush, Adams and Levin have used Raman spectroscopy to monitor and clarify the concerted structural rearrangements arising within the headgroup, interfacial and hydrocarbon regions of the membrane bilayer upon sterol or water addition. For example, the progressive changes involving both intra- and intermolecular rearrangements were followed during the addition to the bilayer of <1, 1, 2, 4 and >4 molecules of water per lipid molecule. In particular, low hydration levels (<2 molecules of water) confer a mobility to the headgroup, glycerol and carbonyl moieties. Conformational changes within specifically the polar headgroup occur on addition of 4 molecules of water, after which no further headgroup or lattice changes occur on continued hydration.

Structural features involving chain packing and headgroup conformational arrangements for bovine brain cerebrosides were investigated by Bunow and Levin. The effects on chain packing by the 2-hydroxy group of the acyl chain of phrenosin and the degree of overlap of the long acyl chains across the central plane of the bilayer were elaborated.

Adams and Levin characterized the gel and liquid crystalline states of didodecyldimethyl ammonium bromide by infrared and Raman spectroscopy. Since this simple organic compound forms membrane bilayers whose headgroup and interfacial regions differ dramatically from normal membrane lipids, comparisons to phospholipid systems allow one to clarify the manner in which specific portions of the lipid molecule stabilize the membrane bilayer.

Jao and Levin developed a Raman spectroscopic technique for distinguishing between the outer and inner monolayers of the egg yolk lecithin membrane. Temperature profiles indicate that the outer monolayer undergoes its gel-liquid crystalline phase transition at about 1°C higher than the inner monolayer.

Exposure to hypoxia (reduced barometric pressure or cardio-pulmonary disease) adversely affects reproductive physiology. Altland and Rattner have found in mice that plasma luteinizing hormone (LH) concentrations were significantly reduced at an oxygen pressure (pO_2) of 131 Torr, whereas growth hormone (GH) and follicle stimulating hormone (FSH) concentrations were only moderately reduced at a pO_2 as low as 86.4 Torr. These changes, which were interpreted as adversely affecting reproduction, were not related to dietary changes, systemic hypothermia or changes in plasma corticosterone (B), but rather to the direct effects of hypoxia, *per se*, on endocrine function. It was also found in an aging study, that tolerance of hypoxia in immature rats was best gauged by the severity of induced hypothermia and the elevation of plasma B concentrations, and the tolerance of young adult (age 140 days) and old rats (age 600 days) by changes in the activity of plasma aspartate aminotransferase.

In collaboration with DCRT, Jennings has made extensive preparations to upgrade, simplify and extend the laboratory computer system in Building 2. New standard modular data collection terminals have been built to service six instrument installations and to exchange data with a new central laboratory computer. It is expected that our ten-year-old central laboratory computer will be replaced during the summer of 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29000-08-LCP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Nuclear magnetic resonance: New methods and molecular structure determination

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

NMR studies of natural and synthetic DNA fibers in the B form showed that significant variations exist in the phosphodiester backbone.

NMR studies of molecules dissolved in liquid crystal solvents in the nematic phase have provided accurate and detailed information about intramolecular geometry in phenazine, N-methylimidazole and phenylsilane.

NMR parameters of ^{57}Fe have been measured in order to characterize this infrequently studied nucleus and facilitate its use as a structural probe. The chemical shift range to be expected in various compounds has been defined, and the mechanisms of spin-lattice relaxation of a ferrocene derivative have been established.

PHS-6040

Project Description:

Objectives:

1. Elucidation of the forces within and between molecules, especially those of potential biological importance.

2. Development of spectroscopic methods for studying molecular structure and analyzing materials of chemical and biological interest.

Major Findings and Future Plans:

DNA. There is a rapidly growing interest in the structure and dynamics of DNA since the discovery of the nucleosome core particle as an elemental unit of protein-DNA in chromatin. We have approached this system by two different NMR methods: one combines solid-state ^{31}P NMR with X-ray diffraction from highly oriented DNA fibers; the other involves high resolution ^{31}P NMR studies of DNA and nucleosome core particles in solution. Recently we have extended the study of the latter system to ^{13}C NMR, which provides more detailed information about conformations and dynamics of DNA in nucleosome core particles. During the current year the greatest emphasis has been placed on studies of DNA fibers, which complemented the solution studies reported last year. In agreement with those earlier studies, the investigations of natural DNA fibers indicated significant variations in backbone conformation along the molecule, and studies of synthetic poly(dAdT)·poly(dAdT) in the B form demonstrated two distinct conformations in the phosphodiester backbone. However, this synthetic polymer in the A form showed only a single uniform backbone conformation. (Shindo)

Oriented molecules. A research program on NMR spectroscopy of oriented biologically important molecules has been initiated. For such a purpose, the work on the structure and conformation of relatively large organic compounds containing nitrogen heteroatoms and small biologically significant molecules has been undertaken. The structure of the tricyclic compound phenazine has been determined. It has been observed that performing the experiments under different orientation conditions substantially increases the information obtainable from the spectra. This idea has been applied to the determination of structure and conformation of N-methylimidazole. It is found that the preferred conformtion of the molecule is the one where one C-H bond of the methyl group lies in the ring plane pointing away from the C=CH bond--information which cannot be easily derived by other techniques. Likewise, in phenylsilane the preferred conformation has been established: one hydrogen of the SiH_3 group lies in the plane of the ring; which contrasts with the carbon analog toluene, where all hydrogens are out of the plane. Differences in charge polarization in CH and SiH bonds can account for these conformational preferences. (Khetrapal)

NMR techniques: The measurement of NMR relaxation times provides much useful information on molecular structure and molecular motion. However, experimental determination of such data, especially T_1, the spin-lattice

relaxation time, is often a time-consuming experiment. In collaboration with Weiss, Ferretti and Gupta, we have been exploring means to minimize the random errors associated with such studies, as well as investigating novel approaches to such measurements. Initial studies, reported last year, were limited to the situation of "ideal" radio frequency pulses--the optimum experimental arrangement. We are now exploring alternative mathematical treatments for dealing with situations often encountered in practice in which limited RF power and/or imperfections in NMR probe geometry must be taken into account. One commonly employed procedure--casting data into a logarithmic form, which can be plotted linearly--has been found to offer little advantage. (Becker)

^{57}Fe NMR. A new project undertaken this year is the development of ^{57}Fe NMR as a new method of chemical structure analysis in biomedical research. Although ^{57}Fe has a nuclear spin I = ½, like that of the proton, it has an extremely low sensitivity, and its NMR has not been explored. Only recently was the first detection of ^{57}Fe NMR reported by German workers. A probe has been built in the LCP which is useful for detection of ^{57}Fe NMR. Current studies are focused on the measurement of the ^{57}Fe chemical shift range and exploration of other intrinsic features of the nucleus, such as its characteristic spin-lattice relaxation time (T_1). A study of the mechanism of relaxation in a ferrocene derivative is nearly complete. Both preliminary T_1 and chemical shift data indicate that ^{57}Fe NMR will be a useful new research probe. We plan to extend these studies to biological systems within the next year. Construction of an improved NMR probe is also planned to coincide with the arrival of the new 500 MHz spectrometer. (Gansow, Shindo)

Publications:

Simpson, R. T. and Shindo, H.: Conformation of DNA in chromatin core particles containing poly(dAdT)•poly(dAdT) studies by ^{31}P NMR spectroscopy. Nucl. Acids Res. 7: 481-492, 1979.

Shindo, H.: Relaxation processes of ^{31}P NMR in macromolecules. Biopolymers 19: 509-522, 1980.

Shindo, H., McGhee, J. D. and Cohen, J. S.: ^{31}P NMR studies of DNA in nucleosomal core particles. Biopolymers 19: 523-537, 1980.

Simpson, R. T. and Shindo, H.: Conformations of 145 base-pair length poly(dGdC)•poly(dGdC) in solution and in association with histones. Nucl. Acids Res. 8: 2093-2103, 1980.

Shindo, H., Wooten, J. B., Pheiffer, B. H. and Zimmerman, S. B.: Non-uniform backbone conformation of deoxyribonucleic acid indicated by phosphorus-31 nuclear magnetic resonance chemical shift anisotropy. Biochemistry 19: 518-526, 1980.

Shindo, H. and Zimmerman, S. B.: Sequence-dependent variation in phosphodiester backbone geometry of a synthetic DNA fibre. Nature 283: 690-691, 1980.

Niu, C-H., Matsuura, S., Shindo, H. and Cohen, J. S.: Direct observation of peptide exchange by stable isotope enrichment. J. Biol. Chem. 255: 2036-2038, 1980.

Tsai, S-C., Shindo, H., Manganiello, V. C., Adamik, R. and Vaughan, M.: Products of reaction catalyzed by purified rat liver guanylate cyclase determined by ^{31}P NMR spectroscopy. Proc. Natl. Acad. Sci., USA. In press.

REPORTED IN PRESS - 1979

Shindo, H., Simpson, R. T. and Cohen, J. S.: An alternating conformation characterizes the phosphodiester backbone of poly(dAdT) in solution. J. Biol. Chem. 254: 8125-8128, 1979.

Becker, E. D., Ferretti, J. A. and Gambhir, P. N.: Selection of optimum parameters for pulse Fourier transform NMR. Anal. Chem. 51; 1413-1420, 1979.

Weiss, G. H., Gupta, R. K., Ferretti, J. A. and Becker, E. D.: The choice of optimal parameters for measurement of spin lattice relaxation times. I. Mathematical formulation. J. Mag. Res. 37: 369-379, 1980.

Becker, E. D., Ferretti, J. A., Gupta, R. K. and Weiss, G. H.: The choice of optimal parameters for measurement of spin-lattice relaxation times. II. Comparison of saturation-recovery, inversion-recovery and fast inversion recovery experiments. J. Mag. Res. 37: 381-394, 1980.

Gupta, R. K., Weiss, G. H., Ferretti, J. A. and Becker, E. D.: A variable perturbation method for nuclear spin-lattice relaxation measurements. J. Mag. Res. 35: 301-306, 1979.

Raiford, D. S., Fisk, C. L. and Becker, E. D.: Calibration of methanol and ethylene glycol nuclear magnetic resonance thermometers. Anal. Chem. 51: 2050-2051, 1979.

Niu, C-H., Bertrand, R. D., Shindo, H. and Cohen, J. S.: Cross-peptide bond ^{13}C-^{15}N coupling constants by ^{13}C and J cross polarization ^{15}N NMR. J. Biochem. Biophys. Methods 1: 135-143, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29001-08-LCP

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Molecular dynamics and vibrational characteristics of membrane assemblies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Vibrational Raman and infrared spectroscopic transitions are used to probe the dynamical and conformational properties associated with lipid-lipid and lipid-protein interactions in membrane bilayer systems. In particular, the concerted interactions of both intrinsic and extrinsic bilayer components upon the three distinct molecular regions of a membrane were examined. Thus, the interrelated perturbations of the headgroup, interfacial and acyl chain regions from cholesterol (7-30 mole %) and water (0.3-4 molecules) were studied both individually and synergistically.

In clarifying the dependence of lipid-protein interactions on acyl chain saturation, membrane recombinants of the myelin proteolipid apoprotein with saturated and unsaturated bilayer matrices were examined by Raman spectroscopy. As a consequence of packing arrangements and diffusion properties characteristic of the two classes of lipids, the apoprotein significantly affects the inter- and intramolecular order parameters of saturated lipids, while only slightly affecting those for the unsaturated bilayers.

PHS-6040
(Rev. 10-76)

Project Description

Within the project area of the vibrational dynamics of membrane components and membrane assemblies our objectives are to probe, clarify and interpret the vibrational spectroscopic characteristics of biological membranes and related systems in terms of inter- and intramolecular order parameters. The general approaches include: (a) analyses of the Raman and infrared spectra of selected systems, and (b) the determination of reliable intramolecular force fields when applicable.

A variety of laser Raman and infrared spectroscopic procedures provide the required data for the various vibrational analyses. The intramolecular force field computations are necessarily performed on a large digital computer.

The studies during the past year were partitioned into roughly two separate areas; first the clarification of specific membrane bilayer processes as reflected by the vibrational spectra of a variety of model membrane assemblies; and second, the continued development of techniques for reliably determining potential functions for moderate size molecules. These investigations are described in the following paragraphs.

1. In describing the dynamical and conformational properties of lipids and proteins in reconstituted biological membranes, recent ESR, NMR and scanning calorimeter studies have suggested the existence of discrete lipid boundary regions surrounding the intruding bilayer component. These boundary lipids are critical for the functioning of the protein in real membrane systems. For the first time we have obtained evidence on the vibrational time scale (10^{-13}/sec) for the existence of a layer of immobilized lipid around an intrinsic membrane component. In particular, the interaction of melittin, a polypeptide consisting of 26 amino acid residues, with dimyristoyl phosphatidylcholine (DMPC) bilayers was investigated by vibrational Raman spectroscopy. Spectral peak height intensity ratios, involving vibrational transitions in both the 3000 cm^{-1} acyl chain methylene carbon-hydrogen stretching mode region and the 1100 cm^{-1} acyl chain carbon-carbon skeletal stretching mode interval, served as temperature profile indices for monitoring the bilayer order-disorder processes. For a lipid:melittin molar ratio of 14:1 two order-disorder transitions were observed. In comparison to a gel to liquid crystalline phase transition of 22.5°C for the pure lipid, the lower transition, exhibiting a 2°C width, is centered at 17°C and is associated with a depression of the main lipid phase transition of dimyristoyl phosphatidylcholine. The second thermal transition, displaying a 7°C interval, occurs at approximately 29°C and is associated with the melting behavior of approximately seven immobilized boundary lipids which surround the inserted hydrophobic segment of the polypeptide. For the lipid:melittin molar ratio of 10:1 two thermal transitions are also observed at 11 and 30°C. As before, they represent, respectively, the main gel to liquid crystalline phase transition and the melting behavior of approximately four boundary lipids attached to melittin. From these data alternative models were suggested for disposing the immobilized lipids around the hydrophobic portion of the polypeptide within the bilayer.

Melittin was also examined in lipid matrices composed of DMPC-d_{54} (all acyl chain methylene hydrogens are deuterated) and mixed matrices of DMPC-h_{54} and DMPC-d_{54}. The two order-disorder transitions are also found in the bilayer matrices of deuterated lipids, but at slightly lower temperatures. Further, the polypeptide prefers a boundary layer composed of deuterated lipids in the mixed bilayer systems. For the DMPC-d_{54} + DMPC-h_{54} + melittin bilayers, three classes of lipids are found: (1) immobilized boundary lipid composed of deuterated molecules, (2) bulk bilayer lipids composed of strongly perturbed deuterated lipids, and (3) bulk lipids composed of slightly perturbed non-deuterated lipids (Lavialle and Levin).

2. Using the myelin proteolipid (or Folch-Pi) apoprotein (mol. wt.: approx. 64-80,000), the dependence of lipid-protein interactions on acyl chain saturation was investigated by Raman spectroscopy. In particular, recombinants of dimyristoyl phosphatidylcholine (DMPC) a saturated bilayer system, and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) an unsaturated bilayer system, with myelin proteolipid apoprotein prepared in an aqueous medium were studied. Peak height intensity parameters, involving vibrational transitions in both the 3000 cm^{-1} acyl chain methylene carbon-hydrogen (C-H) stretching mode region and the 1100 cm^{-1} carbon-carbon (C-C) stretching mode region, were used to construct temperature profiles reflecting bilayer inter- and intrachain order-disorder processes. For liposomes reconstituted with DMPC, the gel-liquid crystalline phase transition T_m, monitored by the C-C stretching mode indices, is depressed from 23°C for the pure system to 12°C and broadened from less than 1°C to about 9°C. On completion of the phase transition at 15°C, the intramolecular chain disorder is substantially greater compared to the pure bilayer form. In addition, no further development of gauche conformers along the chain is apparent as the temperature increases. For the temperature profile derived from the C-H stretching region parameters of DMPC, the phase transition temperature is shifted from 23°C to 11°C. The intermolecular disorder in both the gel and liquid crystalline states is significantly greater in the recombinant systems in comparison to the pure liposomes. In contrast, temperature profiles obtained for recombinants prepared with unsaturated phospholipid bilayers (POPC) indicate that the apoprotein only slightly perturbes the inter- and intramolecular parameters describing the lipid matrix.

In order to assess the rather different perturbations of the apoprotein on bilayers containing either saturated or unsaturated acyl chains, the pure DMPC and pure POPC temperature profiles were compared by matching the curves to the same relative temperature with respect to their values for T_m. That is, the systems are compared under the same environmental conditions. For the liquid crystalline state the POPC bilayers possess a greater population of gauche rotamers along the chain, although, surprisingly, the lateral chain order is increased in the unsaturated bilayer compared to the saturated DMPC system. The increased intermolecular disorder exhibited by the pure and reconstituted DMPC systems was related to the diffusion properties exhibited by saturated and unsaturated lipid matrices.

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4-68

Within the precision of the Raman experiment, no evidence on the vibrational time scale exists for a boundary lipid in the liquid crystalline state involving the aqueous solution of the apoprotein in either the saturated or unsaturated bilayer assembly (Lavialle and Levin).

3. In characterizing the changes occurring within the interfacial region of the lipid molecules forming the bilayer, studies involving the acyl chain carbonyl modes were continued. Infrared and Raman spectra were obtained for the 1690-1770 cm^{-1} carbonyl stretching mode region for 1,2-dipalmitoyl phosphatidylcholine (DPPC) bilayers in the anhydrous, partially hydrated and completely hydrated states. Spectral features at approximately 1740 and 1721 cm^{-1} are assigned to C=O stretching modes associated with the 1- and 2-chain carbonyl groups, respectively. Splittings of the primary transitions at 1743, 1738, ∿1731 and ∿1721 cm^{-1} are attributed to rotational isomers involving the entire chain. Hydrogen bond formation between the fatty acid carbonyl and 3β-OH cholesterol groups was investigated for anhydrous DPPC bilayers. Examination of frequencies, intensities and half-widths of the carbonyl bands indicates that no hydrogen bonding occurs at either of the two carbonyl sites. However, the addition of cholesterol to completely hydrated DPPC dispersions reduces the conformational inequivalence between the two fatty acid carbonyl groups by specifically perturbing the 2-chain. For cholesterol containing systems the carbonyl stretching mode transitions were also used to monitor lattice effects within the interface region as water binds to the bilayer head groups. Specifically, the addition of approximately 2 molecules of water per lipid molecule orders the lipid lattice and increases the bilayer packing density, while the subsequent addition of 4 molecules of water per lipid molecule releases the packing constraints within the interface region and thereby decreases the packing density (Bush, Levin and Levin).

4. Since the conformational and dynamical behavior of biological membranes reflect the concerted interactions of both intrinsic and extrinsic bilayer components, the origins of structural rearrangements within the lipid matrix are often difficult to resolve in liposomal systems. In particular effects from either lipid headgroup or acyl chain perturbations may be propagated throughout the molecule leading to structural reorganizations of the entire phospholipid system. In order to assess the membrane changes that are usually involved, vibrational Raman spectroscopy was used to investigate the conformational behavior of dipalmitoyl phosphatidylcholine (DPPC) bilayers perturbed by cholesterol and water, two membrane components whose lipid interactions involve different regions of the bilayer matrix. Upon addition cholesterol, an intrinsic membrane constituent, to an anhydrous bilayer in concentrations varying from 7 to 30 mole %, modifications in lateral chain interactions were observed by monitoring spectral changes in the methylene C-H stretching and the CH_2 deformation regions. The perturbation in the 1460 cm^{-1} region was not spectroscopically observed until after the addition of 7 mole % of the sterol. Although chain-chain interactions are altered, no additional trans/gauche isomerization is developed along the hydrocarbon chains. Water, a peripheral bilayer component, was added sequentially to the multilayer assembly in the hydration range of 0.3 to ∿4 molecules of water per lipid

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molecule. Vibrational spectra characteristic of motions in the headgroup, interfacial and acyl chain regions of the lipid bilayer were observed. These data indicate that hydration confers a mobility to the headgroup, glycerol and carbonyl moieties. Shifts in the CN symmetric and PO_2^- antisymmetric stretching modes, occurring on addition of ∿4 molecules of water, indicates a conformational rearrangement within the polar headgroup. After ∿4 molecules of water are added to the DPPC system, the spectral features of the gel system [70% (w/w) water] indicate that no further headgroup changes nor increases in either acyl chain trans/gauche or lattice disorder arise on further hydration (Bush, Adams and Levin).

5. Since little structural information exists for glycosphingolipids in bilayer systems, vibrational Raman spectra of the solid and gel phases of bovine brain cerebrosides, kerasin and phrenosin were examined in order to describe the chain packing and headgroup conformations assumed by cerebrosides within the lipid matrix. The carbon-carbon stretching mode profiles (1150-1000 cm^{-1}) indicate that at 22°C the alkyl chains assume an almost all-trans arrangement. These spectral data, combined with those from the C-H stretching region (3050-2800 cm^{-1}), show that phrenosin forms the most highly ordered polycrystalline solid and kerasin the most ordered gel phase. The conformation of the unsaturated, 24-carbon acyl chains is monitored independently by a skeletal stretching mode at 1112 cm^{-1}. The alkyl chains in the kerasin and phrenosin gels are sufficiently extended to allow interdigitation of the 24-carbon acyl chains across the midplane of the bilayer.

The amide I vibrational mode occurs at a lower frequency in solid phrenosin than kerasin, a shift consistent with stronger hydrogen bonding. This band is broadened and shifted to higher frequencies, however, in the phrenosin gel phase. In both the solid and gel phases natural cerebroside exhibits a composite amide I mode.

The disruptive effects on cerebroside chain packing and headgroup orientation arising from the addition of dimyristoyl phosphatidylcholine (DMPC) were examined. Vibrational data for cerebrosides were also compared to those for ceramide, sphingosine and distearoyl phosphatidylcholine (DSPC) structures. Spectral interpretations were discussed specifically in terms of calorimetric and x-ray structural data (Levin and Bunow).

6. In order to clarify the molecular origins of the 34°C thermal pretransition and its effect on acyl chain intramolecular disordering processes in bilayer assemblies, Raman spectra of DPPC gels at 20°C and 34°C were compared in the 1000-1200 cm^{-1} skeletal C-C stretching region. In addition to an overall intensity decrease associated with a change in chain packing characteristics, the growth of intensity in the 1080-1090 and 1122 cm^{-1} regions in the (34°C-20°C) difference spectrum clearly indicates that the thermal pretransition is accompanied by an increase in the population of hydrocarbon chain gauche rotamers toward the center of the bilayer (Bush and Levin).

7. In addition to the boundary lipid surrounding an intrinsic membrane component described above, a second class of protein immobilized lipid occurs in lipoprotein complexes formed by incubating DMPC vesicle bilayers with the apoproteins of plasma high density lipoprotein (HDL). (Plasma lipoproteins are water soluble lipid-protein complexes. The protein components are termed apoproteins.) Apo A-1 (MW ∿28,000), the major protein of HDL, is thought to form a discoidal particle when incubated with DMPC. That is, apoprotein probably forms an annulus about the perimeter of the lipid bilayer.

Temperature profiles for changes in the C-H stretching region of the Raman spectra for the Apo A-1/DMPC discoidal recombinant were determined. For this case the extrinsic apoprotein increases T_m from ∿22°C to 26-27°C. A second order-disorder transition at ∿46°C for only the recombinant system corresponds to the fluidizing of the lipid immobilized by the apoprotein. Electron micrographs and protein assays of our recombinants indicate an average diameter of about 200 Å for the discs, with about 3 or 4 apoprotein molecules per disc. The higher order-disorder transition indicates that 16% of the lipid molecules are immobilized. A calculation, based upon the size of a DMPC molecule, indicates that the apoprotein immobilizes the outer annular ring of lipid forming the disc (Bush and Levin).

8. Didodecyldimethyl ammonium bromide (DDA-Br), a simple organic compound, is capable of forming single shell bilayer vesicles in aqueous media. In comparison to the usual bilayer lipids, this system has a small polar headgroup, no interfacial region and equivalent hydrocarbon chains. Thus, when compared to other membrane lipids, interactions of membrane components with DDA-Br allows a clarification of the efficacy of the various portions of a lipid molecule toward stabilizing the bilayer system. Investigations of the infrared and Raman spectral characteristics of the gel and liquid crystalline states of DDA-Br will be discussed in the annual report of Dr. R. G. Adams (Adams and Levin).

9. In order to understand the fundamental physical and chemical properties of bilayer membranes, it is necessary to distinguish between the outer and inner monolayers of the bilayer unit. Further, it is desirable to avoid, for example, the perturbing effects which arise from either bulky spin labels or NMR shift reagents. We have found that approximately 4 mole % diarachidonyl phosphatidylcholine (DAPC) goes exclusively into the outer monolayer of egg yolk phosphatidylcholine single shell vesicles without perturbing the lipid-lipid interactions inherent in the monolayer. By monitoring changes in the Raman intensity of the double bond feature characteristic of DAPC, it is possible to probe effects on the π electron density arising from conformational rearrangements strictly within the outer monolayer. For the egg yolk lecithin vesicles the sharp transition for the outer monolayer (-13.2°C) is about 1°C higher than the broadened transition centered at -14.0°C which reflects a weighted average for the phase transition over both monolayers. The broadening of the entire profile probably arises from the broadening of the gel-liquid crystalline curve for only the inner monolayer (Levin and Jao).

10. Fluorocyclopropenyl cations are simple Hückel aromatic systems (4n+2, n=o) in which fluorine substitution is expected to exert a marked influence. Since changes in the strength of the ring bonds are reflected in force constant changes, a normal coordinate analysis for the difluorocyclopropenyl cation and a deuterated analog was performed.

The difluorocyclopropenyl cations, $C_3F_2H^+$ and $C_3F_2D^+$, were prepared by reaction of 1,3,3-trifluorocyclopropene-$\underline{d}_0$ and -$\underline{d}_1$ with BF_3 in SO_2 solutions. Raman spectra of these solutions were recorded at -25°C, after proton and ^{19}F nmr spectra had verified a C_{2v} structure for the $C_3F_2H^+$ ion. For use in infrared spectroscopy at -196°C, polycrystalline deposits were prepared by reaction of layers of the trifluorocyclopropenes with BF_3 or SbF_5. The fundamental vibrational frequencies of the $C_3F_2H^+$ ion are, in cm^{-1} ($\underline{a}_1$) 3126, 1945, 1393, 843, 311; ($\underline{a}_2$) 650; ($\underline{b}_1$) 1541, 1025, 924, 583; ($\underline{b}_2$) 924, 366. With these assignments and those for the $C_3F_2D^+$ ion a normal coordinate analysis in a non-redundant internal coordinate representation gave a 13-parameter potential function. Potential energy distributions show that, although CF stretching is spread over several normal modes, most are dominated by single symmetry coordinates. Of particular note is the high frequency of $\nu_7(\underline{b}_1)$, which is largely antisymmetric CF stretching. As expected, the force constant for the CC bond between the fluorine-bearing atoms is substantially larger than the one for the CC bonds to the unsubstituted carbon. Both CC force constants are in the range between those of CC single bonds and CC double bonds, which confirms the aromatic character of this ring system (Craig and Levin).

Significance to Biomedical Research and the Program of the Institute

Raman spectroscopy offers a potentially valuable approach toward examining dynamical properties of natural and model membrane bilayer assemblies since no membrane perturbing agent is introduced by the technique. This is particularly important (a) for investigating conformational changes induced in bilayer systems either by changes in temperature or by addition of membrane substituents and (b) for specifically clarifying lipid-lipid and lipid-protein interactions within the bilayer.

Proposed Course of Project

1. Further characterization of the properties of boundary lipids surrounding proteins will continue using deuterated lipid species as markers.

2. Studies will continue on (a) differentiating between the inner and outer monolayers forming the membrane unit and (b) clarifying the conditions leading to lipid and to protein asymmetry in membranes.

3. Studies on the effects of anesthetics on the thermotropic behavior of model membranes will continue. Interest will be particularly focused on the ability of halothane ($CH_3CHBrCl$) to alter the size of the cooperative unit of bilayer lipids undergoing order-disorder phase transitions. The antagonistic effect of high pressures on phase transition characteristics will be examined.

4. Resonance Raman studies on selected proteins and antibiotics in the ultraviolet spectral region will be emphasized.

Publications

Craig, N. C., Lai, R., Penfield, K. W. and Levin, I. W.: Vibrational spectra and a potential function for the difluorocyclopropenyl-d_0 and -d_1 cations. J. Phys. Chem. 84: 899-906, 1980.

Lavialle, F., Levin, I. W. and Mollay, C.: Interaction of melittin with dimyristoyl phosphatidylcholine liposomes: evidence for boundary lipid by Raman spectroscopy. Biochim. Biophys. Acta. In press.

Bush, S. F., Levin, H. and Levin, I. W.: Cholesterol-lipid interactions: an infrared and Raman study of the carbonyl stretching mode region of 1,2-dipalmitoyl phosphatidylcholine bilayers. Chem. and Phys. of Lipids. In press.

Bush, S. F., Adams, R. G. and Levin, I. W.: Structural reorganizations in lipid bilayer systems: effect of hydration and sterol addition on the Raman spectra of dipalmitoyl phosphatidylcholine multilayers. Biochemistry. In press.

Bunow, M. R. and Levin, I. W.: Molecular conformations of cerebrosides in bilayers determined by Raman spectroscopy. Biophysical Journal. In press.

REPORTED IN PRESS - 1979

Craig, N. C. and Levin, I. W.: Vibrational assignment and potential function for trans-diazene (diimide); predictions for cis-diazene. J. Chem. Phys. 71: 400-407, 1979.

Craig, N. C. and Levin, I. W.: Calibrating Raman spectrometers with plasma lines from the argon-ion laser. Applied Spectroscopy 33: 475-476, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29002-07-LCP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Chemistry and biosynthesis of natural compounds, and instrumental methods used in their study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ulrich Weiss	Research Chemist (Scientist Emeritus)	A LCP

COOPERATING UNITS (if any)
James M. Cook, Department of Chemistry, University of Wisconsin, Milwaukee
Alan D. Harmon, College of Pharmacy, University of Houston
James V. Silverton, NHLBI-LC

LAB/BRANCH
Laboratory of Chemical Physics

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In cooperation with Dr. Harmon, the alkaloids of Amoora rohituka (Aphanamixis polystachya, Meliaceae, have been studied further.

Our synthesis of di- and polycyclic cyclopentanoid ring-systems from 3-ketoglutarate and 1,2-dicarbonyl compounds has been extended to novel cyclic structures in cooperation with Dr. Cook; it is now being applied to the synthetic preparation of certain naturally-occurring substances.

X-ray crystallographic studies by Dr. Silverton have established the molecular geometry of palustric acid, a dienic resin acid. The relationship of this geometry to the chiroptical effects of the acid has been examined.

PHS-6040
(Rev. 10-76)

Major Findings, Future Plans

1. The minor alkaloids from Amoora rohituka have been studied further. The chemistry of one exceedingly sensitive base apparently containing an oxiran (ethylene oxide) ring in vicinity to the nitrogen, is particularly intriguing, since this type of structure seems to be unprecedented. However, the lability of the compound has so far prevented a conclusive elucidation of its structure. This work is to be continued in cooperation with Dr. Harmon, as is the study of the azulene pigments of mushrooms of the genus Lactarius.

2. The study of 2-fluoromorphine and -codeine, and of the other morphines and codeines halogenated in positions 1 or 2, is to be resumed in cooperation with Dr. Kenneth L. Kirk (LC-NIAMDD).

3. In cooperation with Prof. J. M. Cook and his students, work on the synthesis of novel cyclopentanoid ring systems from dimethyl 3-ketoglutarate and suitable 1,2-dicarbonyl compounds has been continued. The synthesis of 1,2; 5,6-bis-cyclopentano-bicyclo[3.3.0]octane-tetraone is one step short of completion, and the total synthesis of modhephene, a unique natural sesquiterpene derived from [3.3.3]propellane, is under way.

This work is to be continued, the goals being synthesis of other members of the relatively neglected class of poly-cyclopentanoid ring systems, and total synthesis of natural compounds derived from such systems.

4. X-ray crystallographic studies by Dr. Silverton have established the conformation of palustric acid. This constituent of rosin contains a 1,3-cyclohexadiene ring and differs from levopimaric acid only in the position of the two conjugated double bonds. This latter acid had been one of the key compounds in our study of the chiroptical effects of non-planar conjugated dienes; similar study of the related palustric acid is of great interest.

Publications

Weiss, U. and Edwards, J. M.: The Biosynthesis of Aromatic Compounds. New York, Wiley-Interscience, 1980, 728 pp.

Moriarty, R. M., Paaren, H. E., Weiss, U. and Whalley, W. B.: A quadrant rule for the prediction of chiroptical effects of optically active cisoid dienes. J. Amer. Chem. Soc. 101, 6804-6810 (1979).

Harmon, A. D., Weisgraber, K. H. and Weiss, U.: Preformed azulene pigments of Lactarius indigo (Schw.) Fries (Russulaceae, Basidiomycetes). Experientia 36, 54-55 (1980).

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4-68

Reported in Press - 1979

Weiss, U., Fales, H. M. and Weisgraber, K. H.: Pigments of Elsinoe Species. Part VII. Revised structure of a synthetic intermediate; formation of a dibenzo-2,2'-oxidodiphenoquinone. Justus Liebig's Annalen der Chemie, 914-919 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1-AM-29003-12-LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The biological chemistry of membranes and complex membrane systems of biological interest.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Gerald M. Shean Research Chemist A:LCP

COOPERATING UNITS (if any)
Karl Sollner (Scientist Emeritus) A:LCP

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29005-06-LCP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Asymmetric synthesis; structure, stereochemistry and NMR

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Herman Ziffer	Research Chemist	A LCP
OTHER:	Cleanthis-Demetre Froussios	Visiting Fellow	A LCP
	Harold Banks	Guest Worker (IPA)	A LCP

COOPERATING UNITS (if any)

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Laboratory of Chemical Physics

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Section on Molecular Biophysics

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In earlier work we had shown that a strain of the mold Rhizopus nigricans could be used to enantioselectively hydrolyze a variety of racemic acetates. We suggested a model which enabled one to rationalize these results and have investigated the hydrolyses of several series of compounds in order to compare the experimental results with predictions made using the model. In a series of acyclic esters replacing hydrogens on the methylene adjacent to the carbinol carbon by one, two or three methyl groups, or by a chlorine atom, did not change the configuration of the carbinol formed. However, in two series of cyclic esters (1-indanyl and 1-tetralyl acetate), replacement of the trans-methylene hydrogen by a variety of substituents always inverted the configuration of the carbinol formed.

Several mono- and di-methyl derivatives of 1,2-indane oxide and 1,2,3,4-tetra-hydro-1,2-naphthalene oxide have been prepared. The 1-methyl derivatives were found to undergo a lithium catalyzed rearrangement to form allylic alcohols, while the 2-substituted compounds were unreactive under the same conditions. Unsubstituted 1,2-indane oxide and 1,2,3,4-tetrahydro-1,2-naphthalene oxide rearranged to 2-indanone and 2-tetralone when reacted with lithium salts.

Project Description

The purpose of this study is to develop methodologies that enable one to synthesize chiral compounds and which allow a tentative assignment of the absolute stereochemistry of the resulting substances. In earlier work we have shown that the microbial reduction of a number of aromatic ketones yield alcohols with predictable configurations. While the microbial reduction of a variety of aromatic ketones yielded alcohols in satisfactory yields, some classes of ketones were not reduced by these microorganisms. The inability of Cryptococcus macerans and Sporobolomyces pararoseus to reduce some ketones, and the potential value of a truly catalytic method utilizing enantioselective hydrolyses, have prompted us to examine in detail the scope and limitations of this latter procedure.

The chiral alcohols produced by these two microbial methods are important in pharmacology and molecular biology where chiral compounds are needed in research on receptor sites, and in determinations of the absolute stereochemistries of metabolites, pheromones and a variety of other biologically important molecules.

Earlier we had proposed a model which predicts the configuration of alcohols formed by enantioselective hydrolyses. In order to test this model we have now examined the products formed in the hydrolysis of two series of 2-substituted cyclic esters (1-indanyl acetate and 1-tetralyl acetate) and a series of acyclic substituted phenyl ethyl acetates. The results from these investigations should characterize the "effective sizes" of substituents that can flank a secondary carbinol carbon and thus makes possible a more accurate prediction of the configurations of alcohols formed in these hydrolyses.

We are also investigating metal catalyzed reactions of aryl oxiranes, since we had shown earlier that it is possible to obtain chiral products from these reactions.

Proposed Course of Project

We have systematically investigated the effect of trans-substituents in 2-substituted 1-indanyl and 1-tetralyl acetates and have recently found that the presence of a cis-substituent (an acetoxyl group) did not interfere with the hydrolysis, i.e., a chiral product was obtained. We plan to examine the effect of other cis-substituents on the configuration of the products formed.

The esterase present in R. nigricans has proved to be remarkably specific in producing chiral alcohols and when possible we will try to purify the enzyme and to attempt to attach it to a solid support. In theory it should then be possible to resolve esters as easily as one can currently separate mixtures of esters and alcohols.

In a cooperative project with a group at the Department of Agriculture we are trying to resolve a racemic synthetic insect pheromone in order to assign the absolute configuration of the natural enantiomer.

We plan to continue the study of lithium catalyzed isomerizations of methyl-substituted aryl oxiranes.

Publications

Imuta, M., Kawai, K. and Ziffer, H.: Product stereospecificity in the microbial reduction of α-haloaryl ketones. J. Org. Chem. In press.

REPORTED IN PRESS - 1979

Imuta, M. and Ziffer, H. Synthesis and physical properties of a series of optically active trans-stilbene oxides. J. Org. Chem. 44: 2505-2509, 1979.

Imuta, M. and Ziffer, H.: Novel copper-pyridine catalyzed ring opening reactions of aryl oxiranes: An asymmetric synthesis of (+)-indane-1,2-oxide and (+)-cis-1,2-indanediol. J. Amer. Chem. Soc. 101: 3990-3991, 1979.

Silverton, J. V., Ziffer, M. and Ziffer, H.: Structure and stereochemistry of condensation products from 1-morpholino-1-cycloheptene and methyl vinyl ketones. J. Org. Chem. 44: 3959-3961, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29006-10-LCP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The relationship between the nuclear and electronic configuration of molecules and their spectroscopic and dynamic properties.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elliot Charney	Research Chemist	A:LCP
OTHER:	Donald C. Rau	Senior Staff Fellow	A:LCP
	John A. Schellman	Distinguished Visiting Scientist (IPA)	A:LCP

COOPERATING UNITS (if any)

Che-Hung Lee — Staff Fellow NCI

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Laboratory of Chemical Physics

SECTION

Spectroscopy and Structure

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A. Macromolcular structure, dynamics and polyelectrolyte properties of large biological polymers, in particular, polynucleotides and nucleic acids are being studied by electric-field induced dichroism and birefringence methods.

Objectives:

I. Macromolecular Structure and Dynamics

The orientation of large polymeric molecules in pulsed electric fields and their disorientation when the field is turned off can be studied using monochromatic polarized light. These experiments are designed to obtain either the molecular conformation or structure in solution (from the dichroism, which is the difference between the changes in extinction of the light polarized parallel and perpendicular to the electric field when the molecules are oriented in the field) or the dynamics of intramolecular reorganization and of rotational disorientation (from the decay of the dichroism at the end of the field pulse). Both of these properties are significantly affected by the presence or absence of free charges on the polymer.

Having demonstrated over the past two years that the ionic environment of the biological polyelectrolytes, DNA and the synthetic polynucleotides poly(A) and poly(C), has profound effects on their orientation in electric fields and that at least two factors in this environment, the ionic strength of the counterions and the pH, produce effects explicable in terms of rapidly developing polyelectrolyte theory, we have turned our attention to other environmental factors and to additional quantitative examination of these effects. The object is to use this information both to obtain structural information and additional clues to the mechanism of the electric field orientation. We have found, for example, that while increases in the ionic strength decrease the degree of orientation of these polyelectrolytes in the absence of buffer or in buffers containing monoionic species, when the buffer is a zwitterion which can bind to the charged polyelectrolyte, the orientation is almost independent of ionic strength and, in fact, has the inverse behavior (from unbuffered or monoionic species buffered) as predicted by a recent theoretical analysis of Manning [Quart. Rev. Biophys. 11: 29 (1978)] of the polarizability of polyelectrolytes. We have also found that the effect of having a divalent counterion such as Mg^{++} present in solution rather than the monovalent Na^{+} is exactly as predicted by this theory. We were encouraged by these results to continue this work in order to provide critical tests of the available polyelectrolyte theories and to develop a new theory for the orientation mechanism based on these.

The first theoretical approach grafted the results of condensation (Manning) =heory on to a first order description of the classical orientation of a dipole molecule in an electric-field. This simple approach satisfactorily describes the above effects and as well, the effect of perturbing the solvent dielectric constant which is explicitly given by the theory.

II. Experimental Research

(1) The effect of the bulk solvent dielectric constant on the polarizability of a highly charged polyelectrolyte was examined by measuring the electric dichroism of low molecular weight DNA in low salt, water-ethanol mixtures. The experimental result, a marked decrease in the orientation with

decreasing dielectric constant was quantitatively reproduced by the theory. Perhaps even more interesting was the direct observation of the transformation of B to A form DNA high alcohol concentrations, direct evidence for which had previously existed only in fibres.

(2) The dependence of the orientation on the charge density of a linear polyelectrolyte, also explicitly given by the theory, has been examined by measuring the low-field induced orientation of single-, double- and triple-stranded forms of poly(rA) and its mixtures with poly(rU). The formation of the double- and triple-stranded structures under conditions suitable for the orientation experiments proved to be difficult because of the competition of the poly(rA)-poly(rA) and poly(rA)-poly(rU) structures at low ionic strengths. The electric dichroism results, although still preliminary, appear to accord with the theoretical predictions.

(3) Another method of examining the charge density parameter is to examine the effective charge density which can be varied in mixtures containing both mono- and divalent ions. The results of these experiments have enabled us to discern two different mechanisms for producing a field-induced polarization and orientation and have provided an excellent confirmation of a still more complete theoretical development (vide infra).

(4) In a cooperative project with J. D. McGhee and G. Felsenfeld, Laboratory of Molecular Biology, NIAMDD, we have measured the dichroism of the DNA of the isolated nucleosome, the 10 nm filament of the multinucleosome (chromatasome) and Mg^{++} condensed 30 nm solenoid (chromatin) and have postulated a structure for this biologically significant structure. Details will be found in the report by Gary Felsenfeld.

(5) Relaxation data and static dichroism measurements on a series of DNA molecules of different molecular weight have led to the tentative conclusion that relaxed linear DNA in solution exists as a worm-like rod or supercoil with apparent linear dimensions smaller than that of a B form rod, while retaining the essential B form structural parameters. Further work on this is underway.

(6) The new electric dichroism apparatus reported under construction last year has been completed and provides an order of magnitude improvement in signal to noise as well as an extension of the useful spectral range into the region of the peptide absorption bands between 200 and 225 nm.

III. Theoretical Research

Despite the concordance between the experimental observations and the theory discussed above, it has been clear that at some point this agreement would have to break down. In fact, in two papers published last year, we showed that observations of the ionic strength dependence of the orientation of DNA in the absence of a zwitterionic buffer were not in accord with the theoretical predictions and that the assumption that the charge on the polynucleotides could be represented, as a simple line charge does not hold when, as in double-stranded poly(rC), part of the charge resides in the interior of

the complex. We have now developed a much more complete theoretical treatment of the polarization of a linear polyelectrolyte, which while it still will not account for structural anomalies like those of poly(rC), it does account for the ionic strength dependence of the orientation and the effects of changes in charge density produced by differential condensation of counterions of different charges on the surface of the polyelectrolyte (Na^{+} and Mg^{++}, for example. This new treatment reduces to the earlier one when counterions of only one charge species is present.

Proposed Course of the Project:

In particular, we are now extending the theory of the field-induced orientation of polyelectrolytes to the high field region to examine the validity of the various extrapolations which have been made from low field data. This is of prime importance in the determination of structural parameters. We also plan a study of the structural dynamics of DNA by electric dichroism relaxation measurements of fixed size fragments. We are also hoping to determine definitively the optical anisotropy of DNA, which has so long remained a refractive element in interpreting dichroism and birefringence and light scattering measurements. Research on the structure of chromatin will continue with an attempt to analyze the structure relationship between the proteins (histones H1 to H5) and the DNA.

We will continue with the study of the structural and polyelectrolyte properties of macromolecules.

Publications:

Charney, E.: The role of the ionic environment in the orientation of polynucleic acids in electric fields. Biophys. Chem. 11: 157-166 (1980).

Charney, E., Lee, C-H. and Rosenfield, J. S.: Calculations of rotatory strengths in chiral chromophores: Sensitivity of structural parameters in dienes. J. Am. Chem. Soc. 99: 3209-3214 (1980).

Lee, C-H., Charney, E. and Tinoco, I. Jr.: Conformation of dinucleoside phosphate in aqueous solution. Biochemistry 18: 5636-5641 (1979).

Charney, E., Yamaoka, K. and Manning, G. S.: Ionic strengths and counterion replusion as factors in the behavior of polyions in orienting electric fields. Biophys. Chem. 11: 167-172 (1980).

Charney, E. and Lee, C-H. Polyelectrolyte orientation in electric fields: The effect of the dielectric constant. Macromolecules 13: 66-68 (1980).

REPORTED IN PRESS - 1979

Charney, E.: The molecular basis of optical activity. John Wiley-Interscience, 1980, 364 pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1-AM-29007-09-LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure and interaction of biomolecules.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hideo Kon	Research Chemist	A:LCP
OTHER:	Fumihide Inoue	Visiting Fellow	A:LCP
	Sumihare Noji	Visiting Fellow	A:LCP

COOPERATING UNITS (if any)
K. M. Smith (Univ. of California, Davis)
M. Chikira (Tohoku University, Japan)
R. W. Hendler (H:LB)

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SUMMARY OF WORK (200 words or less - underline keywords)

Our ESR study of dimerization of metal porphyrins, which covered so far, a number of Cu(II) and Fe(III) complexes, is now extended to a Fe(II) complex. The result is especially significant in view of the role played by binuclear complexes in dioxygen activation in biological systems.

We have started application of spin labeling ESR method to the study of red blood cell deformability in an attempt to obtain rheological information of the cell. Preliminary results show some discrepancy from the behavior projected from simple fluid mechanical arguments, and call for futher investigation.

PHS-6040

Major Findings:

1. Dimer formation of Fe(II)NO-porphyrins: Continuing from 1979 report, the study of dimer formation of metalloporphyrins has been extended to Fe(II) complexes; specifically, NO-liganded Fe(II)(meso-nitro-octaethylporphyrin) was found to form a well-defined dimeric species in concentrated organic solvents. The conclusion was based upon the concentration dependent nature of the ESR (electron spin resonance) signals as well as successful observation of the so-called $\Delta M = 2$ ESR signal having a hyperfine pattern which is interpreted to originate from two ^{14}N nuclei.

This binding enabled us to make an assignment of hitherto unexplained ESR signals observed in NO-liganded Fe(II) hemoglobin when the protein structure is perturbed by salicylate in a certain concentration range of the latter; namely, we can conclude now on the basis of the spectral characteristics that salicylate perturbation causes dimer formation of NO-heme moieties. It is known for a long time that oxyhemoglobin exhibits an enhanced oxygen up-take in the presence of a proper substrate when a certain amount of salicylate is added (oxidase-like activity). The mechanism has never been clarified. Our finding in the model system strongly suggests that similar dimer formation is responsible for the activation of O_2 in oxyhemoglobin, and points to a direction for further investigation of O_2 activation by perturbed hemoproteins.

3. Spin label study of red cell deformability:

High deformability of mammalian normal red blood cells is a vitally important factor in the physiologic function of the cell. We have applied spin labeling ESR method to study the relation of cell deformability and the decay behavior of the bulk magnetic anisotropy of the flow oriented cells when the flow is suddently interrupted. The cell deformability was modified to a various extent by chemically cross-linking cytoskeletal proteins.

From the fluid mechanical considerations of a viscous suspension, it is expected generally that the decay process of magnetic anisotropy by disorientation of the cells would be slower in less deformable cells. However, experiments, at both high and low hematocrit values, demonstrated that the cross-linked uells show considerably faster decay rates than in the intact cells, suggesting the importance of elasticity of cell membrane in determining the decay rate.

Proposed Course of Project:

The ESR study of metalloproteins and the model systems which we have been persuing in the past will remain to be the subject of our potential interest in this project. However, we will shift the emphasis of our effort to the spin label study of red cell deformability at least for the next two years. Our immediate objective is to describe the relaxation of magnetic anisotropy in red blood cells oriented under a shear stress, in more accurate skeological terms, as opposed to the present practice in which deformability and such relaxation are correlated on qualitative basis without a proof. One of the most important item along this line may be to construct a special type of a resonant cavity for

the purpose of generating a uniform shear stress in the red cell suspension. This would make the analysis of the results less ambiguous and, furthermore, reduce the amount of blood specimens required for this experiment.

Publications:

Chikira, M., Kon, H. and Smith, K.M.: Concentration and solvent dependent EPR signals and dimer formation of high-spin Fe(III) porphyrins. J.C.S. Dalton, 526-534 (1980).

Chikira, M., Matsuura, S. and Kon, H.: EPR study of dimerization and conformational change of Cu(II) cytochrome c and its undeca and octapeptide. Biochim. Biophys. Acta, 622: 105-114 (1980).

Weinbach, E.C., Takeuchi, T., Claggett, C.E., Inoue, F., Kon, H. and Diamond, L.S.: Role of iron-sulfur proteins in the electron transport system of Entamoeba histolytica. Arch. Invest. Med. (Mexico) (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29008-09-LCP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Electronic and molecular structural investigations.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ruth McDiarmid	Research Chemist	A:LCP
OTHER:	Abe Auerbach	Staff Fellow	A:LCP

COOPERATING UNITS (if any)

John Doering - Johns Hopkins University

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Spectroscopy and Structure

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The electron impact spectra of butadiene and several substituted butadienes were measured and interpreted. Criteria were established to distinguish between Rydberg and valence transitions. A forbidden valence transition was observed around 7.4 eV in butadiene.

2. The multiphoton ionization spectrum of butadiene was measured to the red of and inbetween the wavelengths previously investigated. Two optically inactive transitions were observed, one of which arises from a 4-photon resonance.

3. A moderately fast pulsed value (speed ∿ 0.2 msec FWHM) was developed as a supersonic nozzle source, tested, and found to work.

PHS-6040

Project Description:

The goals of this research are: (1) To locate and identify the electronic excited states of simple (prototype) polyatomic molecules both to identify the nergetic pathways available to the molecule and to assess the accuracy of the various theoretical techniques and numerical calculations currently employed to describe molecules. (2) To investigate the relation between electronic structure (orbital occupancy) and molecular structure. (3) To study the energy dissipative means available to electrically excited molecules. (4) To develop and employ new techniques for studying the excited states of polyatomic molecules. This project is motivated by the overall philosophy that it is necessary to understand the energetics of the excited states of small molecules before it is possible to explain those of larger more complicated molecules. Energetic problems of the excited states of larger molecules of biological interest include, for example, vision, energy dissipation of uv or radiative excitation, and bond selective chemistry. While we do not pretend to be able to interpret these phenomena, we hope, in our research, to lay a foundation on which such phenomena can be interpreted.

In this research project, several spectroscopic techniques have been employed to locate and identify the excited electronic states of polyatomic molecules. These techniques include ultraviolet (uv) spectrosocpy, electron impact spectroscopy and visible-uv multiphoton ionization (MPI) spectroscopy of static samples. We are currently developing instrumentation to study the MPI spectra of cooled molecular beams. Butadiene, the prototype conjugated molecule, has been the subject molecule for all the investigations reported herein both because it is a molecule about whose excited states a great deal is known (which can be used to interpret new types of spectroscopic data), and because, despite its seminal position in the family of polyenes (leading ultimately to the visual pigments), considerable controversy and uncertainity remains concerning the locations and identification of several of its excited states.

The electron impact investigations reported below were carried out in collabortion with Dr. John Doering of Johns Hopkins University. In these studies, the electron impact spectrum of butadiene has been studied as a function of impact energy and scattering angle both to formulate electron selection "rules" for use in analyzing electron impact spectra and to aid in the analysis of the spectrum of butadiene. We found that the following rules apply to polyatomic molecules: (1) Valence transition have their peak scattering intensity at impact energies close to threshold. (2) Spin allowed, symmetry forbidden transition have little or no intensity in the forward direction and a variable, greater intensity at larger scattering angles. Applying these "rules" to the electron spectrum of butadiene lead to the conclusions that a valence forbidden transition, probably the long sought $^1Ag \leftarrow {}^1Ag$ transition, underlies the Rydberg structure around 7.4 eV and that the 7.06 and 8.00 eV Rydberg transitions belong to the same Rydberg series, the members of which contain considerable valence configuration in their Rydberg "states".

Futher detailed measurement and analysis of the electron spectra of butadiene and various substituted butadienes-- 1,3-cyclo-hexadiene, 1,3-cyclopentadiene,

2,3-dimethyl butadiene, trans, trans-2,4-hexadiene, etc.--lead to the conclusion that the first valence excited state contains Rydberg components and that the magnitude of the mixing depends on the location of the molecule in the valence excited state vibrational manifold. This observation suggests that the reverse relation may be a useful experimental technique--that the admixture of valence and Rydberg configurations in an electronic state may be assessable by an analysis of the incident energy dependence of the plectron scattering intensity of that electronic state.

The multiphoton ionization (MPI) investigations were carried out in collaboration with Dr. Abe Auerbach. The MPI spectrum of static butadiene was studied to confirm that our equipment was working correctly, to extend the MPI spectrum of butadiene into the controversial 1700Å region and to extend uv-vis MPI towards the red to study its relation to IR MPI. The experimental system was shown to function correctly. The MPI spectra of butadiene and butadiene-d_6 showed two new transitions in the 1700Å spectral region. These spectra are now being analyzed. With the aid of Dr. Auerbach's instrumental ability, we were able to develop a fast, high gain amplifier to study resonances too weak to be previously observed. We measured the 4-photon resonant MPI spectrum of one band of butadiene, confirmed that the resonance arose from the simultaneous absorption of four photons, estimated the cross section of the resonance to be $\sim 10^{-110}$, and confirmed that the selection rule observed for this 4-photon resonance was that predicted on theoretical grounds.

To enable these spectra, and others, to be studied at greater molecular resolution, work was simultaneously carried out on the cooled molecular beam system in collaboration with Dr. Auerbach. A commercial pulsed valve was modified for molecular beam use and demonstrated to work correctly. In preliminary experiments, MPI signals were obtained from a molecular beam, but additional work needs to be done on the experimental system before it can be used for experimentation.

In the next year, these studies will all be continued. The "rules" that have been establishex for electron impact spectroscopy will be applied to other experimental systems of fundamental interest, such as non-conjugated dienes, trienes and ethylene. We hope that the cooled molecular beam system will soon be fully functional so that MPI spectroscopy can be carried out on isolated cooled molecules, starting with butadiene. If our purchase request for a quadruple molecules, starting with butadiene. If our purchase request for a quadruple mass spectrometer is funded, we shall also study the dependence of the fragmentation pattern of molecular ions on the excited vibrational state of the multiphoton resonant excited states reached in MPI, and, perhaps, on the first photon resonance in the multiphoton process.

Publications:

Doering, J.P. and McDiarmid, R.: Electron impact study of the energy levels of trans-1,3-butadiene. II. Detailed analysis of valence and Rydberg transitions. J. Chem. Phys. (in press).

Auerbach, A. and McDiarmid, R.: A modified pulsed valve for supersonic jet applications. Review Sci. Instr. (in press).

McDiarmid, R. and Doering, J.P.: Anomalous $V_1 \leftarrow N$ vibrational band intensities in the low energy electron impact spectra of trans-dienes. J. Chem. Phys. (in press).

REPORTED IN PRESS - 1979

McDiarmid, R.: A reinvestigation of the absorption spectra of ethylene and ethylene-d_4 in the vacuum ultraviolet. J. Phys. Chem. 84: 64-70 (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29009-07-LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on sickle cell disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William A. Eaton	Medical Director	A:LCP
OTHER:	James Hofrichter	Research Chemist	A:LCP
	Frank A. Ferrone	Staff Fellow	A:LCP
	Helen R. Sunshine	Staff Fellow	A:LCP
	Massimo Coletta	Guest Worker	A:LCP
	David Felsenthal	Summer Worker	A:LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Macromolecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	2.1	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The thermodynamics and kinetics of hemoglobin S gelation are being investigated in order to elucidate the molecular mechanism for this process. The results of these studies provide the rationale for developing agents that can be used in the treatment of patients with sickle cell disease. A method to measure the distribution of delay times for intracellular polymerization is being developed as an assay technique to test the efficacy of potential therapeutic agents in inhibiting intracellular gelation of hemoglobin S.

PHS-6040

Objectives:

The major aim of this work is to elucidate the molecular mechanism of hemoglobin S gelation in order to provide a rationale for the development of agents that can be used in the treatment of patients with sickle cell disease. Assay techniques are also being developed to test the efficacy of potential therapeutic agents in inhibiting intracellular gelation of hemoglobin S.

Methods Employed:

In addition to conventional biochemical methods, a variety of specifically designed physical techniques have been employed to study the gelation process. These include microspectrophotometry linear dichroism and birefringence, water proton NMR line widths, temperature perturbation kinetics, laser photolysis, and laser light scattering.

Major Findings:

I. Photoinduced Gelation of Hemoglobin S

The principal aim of this study has been to obtain kinetic data over a wide dynamic range and to measure delay times as short as milliseconds. The results of these studies can then be used to test various possible models for the kinetic mechanism of gelation.

The photolysis of the carbon monoxide complex of hemoglobin S by a CW argon laser has been used to rapidly initiate gelation, and the gelation process monitored by measuring the scattered laser intensity. Using this technique, we have completed a set of kinetic measurements over the time range 0.001 to 10,000 seconds, the concentration range 20 to 40 g/dl, and the temperature range 5 to 50°C. This represents the most comprehensive set of kinetic measurements yet made on this system. Three principal new results have emerged from this study, and have suggested a new mechanism for the nucleation and polymerization of hemoglobin S (see Part II of this report).

First, the concentration dependence of the delay time decreases from about 30th power at the longest times to less than 10th power at the shortest times. The time dependence of the delay time also decreases with decreasing delay time, approaching the value expected for a homogeneously nucleating polymerization process. Finally, under conditions where the delay time is less than a second, many domains of polymers are formed in the photolysed volume of about 10^{-9} cc, and the delay times are highly reproducible in repeated experiments. In marked contrast, under conditions where the delay time is longer than several seconds, only a single polymer domain is formed, and the delay times are extremely variable in repeated experiments. Although the delay times are highly reproducible in these slowly gelling samples, the post delay growth rate is the same, suggesting that a single even initiates the onset of a large number of events. The variability in the delay time, then, represents the probability distribution for the occurence of this single event.

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II. Molecular Mechanism of Hemoglobin S Gelation

This study is aimed at providing a theoretical model for the molecular mechanism of gelation that makes quantitative predictions of the kinetic behavior, and yields parameters that can be interpreted in terms of molecular structure.

The results described above have suggested a mechanism in which there are 2 pathways for polymer formation--homogeneous nucleation of polymers in the bulk solution phase, as we have previously proposed, and heterogeneous nucleation of polymers on the surface of existing polymers. At the lowest concentrations, homogeneous nucleation is so improbable that stochastic behavior is observed in the small sample volumes; and heterogeneous nucleation is the dominant pathway for polymer formation, thereby accounting for the high time dependence in slowly gelling samples. At the highest concentrations homogeneous nucleation becomes much more probable, and the time dependence decreases. The decrease in the concentration dependence of the delay time with increasing concentration results from a decrease in size of both the homogeneous and heterogeneous nuclei.

We have begun to quantitatively test this model for the gelation mechanism by employing an equilibrium nucleation assumption for both homogeneous and heterogeneous nucleation. This assumption permits the calculation of nucleation rates from a calculation of the equilibrium constants for nucleation using statistical thermodynamics. Growth of the polymer is then treated according to standard methods, and the differential equations describing the rate of nucleation and the incorporation of monomer into polymer are then solved numerically using the DEC10 computer. Thus far, a limited region of parameter space has been explored, but it seems clear that the model is plausible and may explain all of the major kinetic observations on the gelation of hemoglobin S.

III. Oxygen Binding by Sickle Hemoglobin Polymers.

The principal objective of this project is to provide a thermodynamic description of the gelation of hemoglobin S solutions partially saturated with oxygen, and, in particular, to measure the oxygen binding curve of the polymer. The gel of deoxygenated hemoglobin S is well described by a thermodynamic model in which an aligned polymer phase is in solubility equilibrium with a concentrated highly non-ideal solution of monomers. The gelation of hemoglobin S partially saturated with oxygen or carbon monoxide can also be understood in terms of this two-phase model. Binding measurements indicate that the solution phase of a gel exhibits a normal oxygen dissociation curve. In order to obtain the binding curve for the polymer phase, we have carried out two types of experiments. In the first, the polymers of a gel partially saturated with xygen are sedimented, and the total saturation of the supernatant is determined by near infrared spectrophotometry. In the second, a well-ordered domain of ligned polymers is formed using a focussed argon ion laser to nucleate elation, and the linear dichroism of the polymers is measured at high sensitivity with a microspectrophotometer by modulating the linear polarization and using synchronous detection.

From these results we calculate a gel (i.e., solution plus polymer) binding curve which agrees well with that measured by Gill et al., confirming the validity of the two-phase model. The polymer binding curve is non-cooperative with a binding constant about 2-1/2 times less than K_T of the two-state allosteric model. The simplest interpretation of this result is that R-state molecules do not copolymerize and that liganded T-state molecules are excluded from the polymer by about 600 calories per ligand bound. Part of the reduced affinity of polymerized hemoglobin S may be understood in terms of the structure of the intermolecular contacts. Residues in the EF corner of the β subunit are most probably involved in a intermolecular contact with the β6 region of a molecule in an adjacent strand of the polymer. Since residues of the EF corner undergo conformational changes upon ligand binding, this intermolecular contact in the polymer could very well stabilize the deoxy tertiary conformation.

IV. Intracellular Gelation of Hemoglobin S.

The laser photolysis - light scattering technique is being developed to investigate the distribution of delay times for intracellular gelation in blood samples under near physiological conditions. Preliminary measurements indicate that the wide distribution of delay times (milliseconds to kiloseconds), expected from studies on purified hemoglobin S solutions, is found in red cells This results is important in several respects. First, it suggests that intracellular gelation takes place by the same kinetic mechanism as extracellular gelation. Second, the observation of subsecond gelation delay times in cells is consistent with the kinetic hypothesis, which requires that intracellular gelation take place in times less than the capillary transit time for some fraction of the cell population. Finally, it suggests that the measurement of the distribution of intracellular delay times could be the most relevant assay for testing the inhibitory effects of potential therapeutic agents.

Significance to Biomedical Research and the Program of the Institute:

Our kinetic studies constitute a totally new approach to understanding the pathophysiology of sickle cell disease and to the development of therapeutic measures.

The theoretical approaches, the experimental results, and the methods developed in this investigation are contributing to a general understanding of other macromolecular self-assembly processes.

Proposed Course of Project:

The new nucleation mechanism will be explored theoretically to establish what range of physical parameters are capable of quantitatively predicting the observed kinetic behavior. Linear dichroism measurements will be carried out on the polymer binding of carbon monoxide under the same conditions as the oxygen binding measurements. The laser photolysis, light scattering technique

will be futher developed and employed to measure the effect of potential therapeutic inhibitors on the distribution of intracellular delay times. The technique will also be used to compare the distribution of delay times with clinical severity.

Publications:

Eaton, W.A.: The problem of inhibiting hemoglobin S gelation in patients with sickle cell disease. In *The Combined Clinical Staff Conference on Scientific Basis of New Therapeutic Approaches in Sickle Cell Anemia*. Annals of Internal Medicine (in press).

Hofrichter, J., Sunshine, H.R., Ferrone, F.A. and Eaton, W.A.: Oxygen binding and the gelation of sickle cell hemoglobin. In *Molecular Basis of Hemoglobin Dysfunction*, P. B. Sigler (Ed.), (Elsevier/North Holland) (in press).

Ferrone, F.A., Hofrichter, J., Sunshine, H.R. and Eaton, W.A.: Kinetic studies on photolysis induced gelation of sickle cell hemoglobin suggest a new mechanism. In *Biophysical Discussion on Proteins and Nucleoproteins: Structure, Dynamics and Assembly*, Biophysical Journal (in press).

Sunshine, H.R., Hofrichter, J., Ferrone, F.A. and Eaton, W.A.: Oxygen binding by sickle hemoglobin polymers. In *Interaction of Iron With Protein in Oxygen and Electron Transport* (in press).

REPORTED IN PRESS - 1979

Sunshine, H.R., Hofrichter, J. and Eaton, W.A.: Gelation of sickle hemoglobin in mixtures with normal adult and fetal hemoglobins. J. Mol. Biol. 133: 435-467 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1-AM-29010-08-LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Conformation and electronic structure of biological molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William A. Eaton	Medical Director	A:LCP
OTHER:	James Hofrichter	Research Chemist	A:LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Macromolecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A correlation has been found between the exons of the globin gene and the functional parts of the hemoglobin molecule, suggesting a role for exon-recombination in the evolution of cooperative hemoglobins.

PHS-6040

Objectives:

The aim of this research is to investigate the dynamics of conformational transitions in proteins using time-resolved pulsed laser spectroscopy, and to relate structure and function in proteins.

Methods Employed:

For measurements of transient absorption, linear dichroism and birefringence, luminescence, and Raman scattering an excite and probe type spectrometer is being developed, which is based on two Nd:YAG nanosecond lasers, a dye laser, and an optical multichannel analyzer.

Major Findings:

I. Dynamics of structural changes in hemoglobin.

During the past year, Dr. Hofrichter has developed the transient spectrometer in the absorption mode and has measured high quality isotropic absorption spectra with single laser shots following the complete photolysis of the carbon monoxide complex of hemoglobin (see annual report by Dr. Hofrichter, Z01-AM-29010-08-LCP, for a description of the spectrometer and initial results).

II. Correlation of exons and functional parts of hemoglobin.

Gilbert has suggested that the presence of intervening sequences in DNA, called introns, can speed evolution by allowing novel proteins to be constructed from the pieces of existing ones. This hypothesis further suggests that the coding sequences, called exons, correspond to functional parts of the protein. The most striking example so far is the case of the immunoglobulin γ heavy chain, where the four polypeptide sequences corresponding to the four coding sequences form structurally and functionally distinct parts of the molecule. The relation between the three coding sequences of the β globin gene and structure of function is not as obvious, but the central coding sequence does code for the part of the globin chain which forms the heme crevice. To further test the idea that coding sequences correspond to functional units of proteins we considered the relationship between the coding sequences of α and β globin genes and the corresponding parts of the complete, tetrametic haemoglobin molecule.

First, most of the residues (16 out of 20) in contact with the heme correspond to the central coding sequence of the β globin gene. In the α gene, 16 out of 20 of the heme contact residues are also contained in the central sequence. The distribution of intersubunit contacts among the three coding sequences shows a very strong correlation as well. In the case of the $\alpha_1\beta_2$ contact, the central sequences contain 12 out of 15 residues in the β subunit that form intersubunit bonds, and 13 out of 18 residues in the α subunit. For the $\alpha_1\beta_1$ contact, the third sequence contains 13 out of the 18 intersubunit bonding residues in the α subunit, and 14 out of 18 in the β subunit. The distribution of residues involved in binding the regulators--

protons, carbon dioxide, and DPG--indicates no marked correlation, although the most important residues are contained in the first and third sequences.

Thus, the distribution of functional residues among the 3 coding sequences indicates that coding sequences do correspond to the major functions of hemoglobin. This result would suggest a pathway for hemoglobin evolution in which the current first and third coding sequences arose from replacement of whole sequences in single steps, thereby speeding the evolutionary process. These ideas can be tested from functional studies on combinations of the polypeptide sequences coded for by the 6 exons of the α and β globin genes.

Significance to Biomedical Research and the Program of the Institute:

The elucidations of the dynamics of the structural transitions in hemoglobin should lead to a much deeper understanding of how this and other proteins perform their biological function. The techniques developed in the course of these studies should be applicable to a wide variety of problems in protein physical chemistry.

Proposed Course of Project:

In the coming year, we shall continue to develop the transient laser spectrometer, and begin a detailed investigation of the dynamic of structural changes in hemoglobin.

Publications:

Eaton, W.A.: The relationship between coding sequences and function in hemoglobin. Nature, 284: 183-185 (1980).

Eaton, W.A. and Hofrichter, J.: Linear dichroism of hemoglobin. Methods in Enzymology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1-AM-29011-09-LCP

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Physics and Chemistry of Photoreception

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William A. Hagins	Medical Officer	A LCP
OTHER:	S. Yoshikami	Research Biologist	A LCP
	F. M. Hagins	Guest Worker	A LCP
	H. Kon	Research Chemist	A LCP
	W. G. Banfield	Guest Worker	LP C
	J. George	Biological Laboratory Technician	A LCP
	M. Foster	Guest Worker 1 July 1980-	(IPA)
	S. Baker	Summer Employee 16 June - 1 September 1980	A LCP

COOPERATING UNITS (if any)
W. E. Robinson — Johns Hopkins Hospital, Baltimore, Maryland 21205

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An investigation of the mechanism of visual excitation in vertebrates.

PHS-6040

Project No.: Z01-AM-29011-09-LCP

Project Description:

During 1979 new methods were developed for measuring net movements of Ca^{++} from various parts of the rod cells of vertebrate retinas with the aid of specific Ca^{++}-sensitive microelectrodes placed in the extracellular spaces of the retina under direct observation by infra-red microscopy. By analyzing the concentration gradients of Ca^{++} in light and darkness with these electrodes together with voltage gradients measured with standard fluid-filled microelectrodes a number of new findings have resulted (Yoshikami, Hagins).

1. Ca^{++} diffuses freely in the space between retinal rod cells.

2. The interstitial Ca^{++} concentration in retinas is stabilized by a control mechanism that takes up Ca^{++} or releases it into the interstitial space when the composition of the perfusing fluid is changed.

3. Light causes a large, fast efflux of Ca^{++} from the outer segments of living rods in rat and frog retinas. This efflux is coincident with the light-induced suppression of the dark current that is the primary electrical signal in visual excitation. At low light stimulus intensities at least 500-1000 Ca^{++} ions emerge from each rod for each photon absorbed in its outer segment. The Ca^{++} efflux continues for as much as 1 minute in steady light and can cause rod outer segments to lose as much as 20% of their total Ca^{++} content. The efflux does not require that Na^{+} be present. It persists when the Ca permeability of the rod plasma membrane is increased with divalent ionophores such as X-537A. All of these findings conform with predictions of the hypothesis that the essential step in visual excitation by which light controls the dark current is release of Ca^{++} into the cytoplasm of visual receptor cells.

4. Preliminary experiments have been carried out to find the source of the light-released Ca^{++} by studying the Ca^{++} binding and releasing properties of isolated fragments of rod outer segments. It has been possible to show that concentrated suspensions of fragments possess the property of taking up free Ca^{++} in the dark and releasing it in light with a time course similar to that of visual excitation.

Publications:

Robinson, W. E. and Hagins, W. A.: GTP hydrolysis and the transmitter cycle in visual excitation. Nature (Lond.) 280, 398-400 (1979).

Yoshikami, S., George, J. S. and Hagins, W. A.: Light-induced calcium fluxes from the outer segment layer of vertebrate retinas. Nature (Lond.) (1980) in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1-AM-29012-10-LCP

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The influence of molecular structure on chemical and biological properties

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Norman E. Sharpless	Research Chemist	A LCP
OTHER:	Ralph G. Adams	Research Physicist	A LCP
	William H. Jennings	Research Physicist	A LCP

COOPERATING UNITS (if any)
Peter F. Kador EI LVR, NIH; Che-Hung Lee, C, NIH; and C. H. L. Kennard, Reader in Chemistry, University of Queensland, Brisbane, Australia

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The energy minimization calculations on compounds of biological interest continues to give insight into their modes of behavior, and clues to their pharmacophores. Additional mathematical analysis of retinal isomers confirms the uniqueness of those forms involved in the visual process.

The inhibition of aldose reductase by flavonoids and related compounds is being extended to stereochemical and other QSAR factors.

Project No.: ZO1-AM-29012-10-LCP

Project Description:

This project investigates the molecular and submolecular factors, such as structural features, quantum parameters and physical properties in relation to biological and pharmacological behavior of biologically important substances.

Energy minimization and conformation studies.

Using both the energy minimization program GEMO, as well as molecular orbital studies (HUCKEL and CNDO), the studies of the relative conformations of retinal isomers and analogs have been continued to gain insight into the visual process.

Quantum studies by the CNDO technique, followed by cluster analysis on the electronic parameters so obtained confirm the uniqueness of the 11-cis and the 11,12-s-dicis forms, which are those isomers involved in the initiation of the visual process.

Previous work in this laboratory (Sharpless, Adams, Jennings) has indicated that the number (and position) of the methyl groups in retinene poises the molecules' energy at just that point necessary for the visual process to be activated, if a charge transfer process be postulated. CNDO calculations on a variety of methyl analogs of retinene show a constant increase in energy values (electronic energy, total energy, binding energy) for methyl group attached to the pi system, but independent of the position of that group. However, the dipole moment depends also on the position of the methyl group.

The energy of the lowest empty molecular orbital (and that of the highest occupied molecular orbital) shows a maximum (and minimum) of the isomer containing three methyl groups (retinene itself), in agreement with the charge transfer theory of rhodopsin complexing postulated previously.

Aldose reductase has been implicated in the events which lead to sugar cataracts by its action in forming sugar alcohols, which may be the causes of the cataracts. Flavonoids and related compounds have been reported to be potent inhibitors of aldose reductase. Our postulated mechanism of inhibition is a nucleophilic assault on the carbon atom of a carbonyl group by the reductase, which then becomes unavailable to its usual substrate. Since certain stereochemical requirements must also be met, investigations using sterically non-constrained enantiomers of 1,3-dioxo-1H.benz[de] isoquinoline-2(3H)-2'-propionic acid and the constrained spirohydantoin enantiomers of 6-fluoro-spiro-[chroman-4,4'-imidazolidine]-2',5'-dione showed that aldose reductase recognizes stereochemistry at an inhibition site. Concomitant with these results are our calculations to further the QSAR relations in these compounds by improved quantum calculations, molar refraction calculations and partition coefficients. Quantum calculations have been carried out on a series of benzophenanthrene derivatives with cis/trans hydroxyl and epoxide groups. The data are now being evaluated.

Project No.: Z01-AM-29012-10-LCP

Publications:

Kador, P. F., Goosey, J. D., Sharpless, N. E., Kolish, J. and Miller, D. D.: Stereospecific inhibitions of aldose reductase. Eur. J. Med. Chem. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29013-09-LCP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
The physics and chemistry of the visual pigments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Ralph G. Adams	Research Physicist	A LCP
OTHER:	Norman E. Sharpless	Research Chemist	A LCP
	William H. Jennings	Research Physicist	A LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Model systems of visual pigments are constructed using retinal and retinal isomers in π-π complexes with amino acids and peptides. Models are examined both spectroscopically and with computer programs (GEMO) applications to determine how to approximate rhodopsin.

PHS-6040

Project Description

Optimistic projections of results obtained from tripeptide (l-phenyl-alanyl-l-alanyl-l-lysine methyl ester) were rather premature. It becomes obvious that further development of the proteinaceous portion of rhodopsin requires many hands and I have momentarily set this effort aside.

Computer oriented investigation of the probable structure of rhodopsin has led to the conclusion that methyl groups are a prime factor that help make 11-cis retinal unique as a chromophore for rhodopsin.

Future Work

Tripeptide work will be held in abeyance for the time being.

The computer program is still in progress and current attempts to enlarge the capacity will enable us to expand on our preliminary efforts to model a visual pigment.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1-AM-29014-20-LCP

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effects of environmental stress (hypoxia, exercise, smoke) on animals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul D. Altland	Research Physiologist	A LCP	NIAMDD
Other:	Milton G. Parker	Biological Lab Technician	A LCP	NIAMDD

COOPERATING UNITS (if any)
Barnett Rattner (Guest Worker), Patuxent Wildlife Research Center

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In studies designed to determine reproductive hormone responses of male mice following acute exposure to simulated high altitude (hypoxia), it was found that moderate hypoxia significantly reduced plasma concentrations of luteinizing hormone (LH), and to a lesser degree follicle stimulating hormone (FSH), and growth hormone (GH), even in the absence of changes in dietary intake, systemic hypothermia and the stress related rise in plasma corticosterone concentration (B). Prolonged exposure of mice to moderate to severe degrees of hypoxia depressed plasma LH concentration for 1 day, and plasma FSH and corticosterone for as long as 3 weeks.

In studies designed to determine the altitude tolerance of male rats ranging from 30 to 600 days of age it was found that the critical threshold for survival in immature rats was 8230 m, and for young adult and old rats 7620 m. In immature rats the degree of hypothermia and elevated plasma B concentration were the most reliable criteria for determining altitude tolerance, and in young adult and old rats, changes in plasma aspartate amino transferase activity was the most important criterion for predicting altitude tolerance.

Project No. ZO1-AM-29014-20-LCP

Project Description:

Exposure to reduced oxygen tensions, associated with high altitudes or cardiopulmonary abnormalities, is known to adversely affect reproductive physiology. The endocrine responses associated with such conditions (hypoxia) are not well known. Male mice were exposed for 4 hours to hypoxia (simulated altitudes 1524-7620 m; bp 632-282 Torr; pO_2 131-57 Torr) on concentrations of plasma gonadotropins, GH, Prolactin (PRL) and corticosterone. The peripheral levels of LH in plasma pools (n=12) were consistently reduced ($p<0.05$) by an exposure as low as 1524 m., whereas FSH and GH estimates were somewhat reduced, and plasma PRL concentrations were not uniformly affected, but circulating B levels increased above 4572 m (pO_2 86,4 Torr). The dramatic reduction in plasma concentrations of LH, and the moderate decrease in FSH, represent the direct effects of hypoxia, per se. The animals were deprived of food and water during the treatment period, and thus they were not confounded by differential dietary consumption, a commonly encountered complication in analyzing hormone changes. The hormonal changes also appeared to be independent of the stress evoked alteration in plasma corticosterone and systemic hypothermia. The plasma LH changes were transient (3 Day), but plasma FSH concentrations remained depressed for as long as 4 weeks at 6706 m.

The changes in the plasma concentrations of LH, FSH, GH and B upon exposure to simulated high altitude are due to changes in hormone secretion and possibly clearance. It is known that maximal activation of the hypothalamo-hypophyseal-adrenocortical system during acute hypoxia is dependent upon peripheral chemoreceptors and central adrenergic and cholinergic function. In the rat it has been demonstrated that hypoxia decreases brain synthesis and metabolism of norepinephrine, dopamine, and 5-hydroxytryptamine due to diminished activities of tyrosine and tryptophan hydrolases, which is presumably the result of a reduction in oxygen substrate. These changes in neurotransmitter turnover, and possibly the direct effect of reduced oxygen on hypothalamo-hypophyseal function, may ultimately be responsible for the observed changes in the circulating concentrations of gonadotropins and GH.

It has been known for many years that immature animals are less susceptible to asphyxia and hypoxia than young adults, but the tolerance of aged animals is not well known. Study of this subject is needed since ascent to altitude by people of all ages is more commonplace than in the past, and our knowledge of the effects of hypoxia on man and animals is incomplete. In addition to the use of survival or cessation of a somatic reflex as the terminal point, additional physiological and biochemical data has been used to assess altitude tolerance. It was found that the tolerance of immature rats was greater than the adult rats, and that there was no difference in the survival rate of young adult and 600 day old rats. The degree of hypothermia in immature rats was directly related to hypoxic severity; 33°C was the critical body temperature before death. Body weight loss, and plasma B concentration elevations, also occurred in immature rats at the critical survival threshold. Hypothermia in adult rats was similar at all altitudes. Plasma enzyme activity in immature rats was unchanged at altitude. Plasma aspartate aminotransferase (PAsAT) and lactic dehydrogenase activity in young rats and PAsAT and

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fructose diphosphate aldolase activity in old rats were first elevated at 7620 m, the altitude at which deaths first occurred. This suggests that increased plasma enzyme activity may be useful in predicting relative altitude tolerance. Except for renal pathology in old rats, no other tissue changes were found. In young and old rats changes in PAsAT activity was the most important criterion found for predicting the relative altitude tolerance. The plasma enzyme changes were attributed to the effects of hypoxia on cell membrane permeability.

Publications:

Rattner, B. A., Michael, S. D. and Altland, P. D.: Plasma concentrations of hypophyseal hormones and corticosterone in male mice acutely exposed to simulated high altitude. Proc. Soc. Exp. Biol. Med. In press.

Rattner, B. A., MacMillan, B. T., Michael, S. D. and Altland, P. D.: Plasma gonadotropins, prolactin, and corticosterone concentrations in male mice exposed to high altitude. Jour. Reprod. Fertility. In press.

Altland, P. D. and Rattner, B. A.: Effects of nicotine and carbon monoxide on tissues and systemic changes in rats. Environmental Research 19: 202-212, 1979.

Rattner, B. A., Gruenau, S. P. and Altland, P. D.: Cross-adaptive effects of cold, hypoxia, or physical training on decompression sickness. J. Appl. Physiol.: Resp., Environ., Exer. Physiol. 47: 412-417, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29015-09-LCP

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Digital computer facilities for LCP and LMB

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William H. Jennings, Jr. Research Physicist A LCP

COOPERATING UNITS (if any)
Computer Systems Laboratory, DCRT: Arthur R, Schultz, Jr., John I. Powell, Eugene O'Bryan, Ronald Fico

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The replacement of the laboratory computer facility serving LCP and LMB is nearing completion. A local network now connects five of the seven instruments to be serviced. The new system, a three level hierarchical configuration, provides a dedicated micro computer for each instrument, a store and forward communications processor and a large capacity host computer.

PHS-6040
(Rev. 10-76)

Project No.: Z01-AM-29015-09-LCP

Project Description:

This computer facility has served the laboratories of Chemical Physics and Molecular Biology for the past eleven years. In cooperation with the Computer Systems Laboratory, DCRT, we are replacing the obsolete existing system, a star configuration, with a local network in the form of a three level hierarchical system. This transition has caused a minimum of disruption because the initial implementation of the network uses the same host as the existing system. Upon conversion of all presently connected instruments, the last phase of the replacement operation will be the change to a new host computer.

Objectives:

Development of a laboratory computer facility to service the data acquisition and processing requirements of the laboratories in Building 2.

Findings:

The complete replacement of the Honeywell 516 computer system should be accomplished this year. The new hierarchical system has proven very reliable throughout a year of routine operation and the flexibility gained by the new architecture has been demonstrated in numerous ways. Five of the seven instruments serviced by the 516 have been converted to the new system and the replacement for the Raytheon mini-computer is well along in its development. This year this project has proceeded simultaneously developing the hardware and software elements at all three levels of the system. The work on the communications processor is complete although revised documentation and some enhancements will be added at the time of host changeover. The dedicated instrument microcomputers (LDACS) have a standardized hardware configuration and the data acquisition software developed this year is highly modularized. We anticipate that by the end of this year the software standards will be completed and documented. Although we presently have all hardware components to effect host changeover, that awaits completion of the instrument connections. The communications link to the new host, a DEC 11/70 is completed and running under test on the CSL development 11/70 system. Data processing software is also under test in the LDACS systems as well as the 11/70 system. As indicated in the last annual report, most new data processing software will be available in a tested form upon host changeover.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29016-05-LCP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Macromolecular Dynamics and Assembly Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James Hofrichter	Research Chemist	A:LCP
COLLABORATORS:	William A. Eaton	Medical Director	A:LCP
	Frank A. Ferrone	Staff Fellow	A:LCP
	Helen R. Sunshine	Staff Fellow	A:LCP

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Spectroscopy and Structure

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continuous laser photolysis of samples saturated with carbon monoxide has been used to rapidly prepare concentrated deoxyhemoglobin S samples. Subsequent to deoxygenation, scattering of laser light has been used to monitor the concentration and temperature dependence of the polymerization reaction. Both the concentration dependence of the tenth-time and the time dependence of the process, decrease as the delay time becomes shorter than 100 milliseconds. In addition, in slowly gelling samples, where single domains of polymers are formed in the observed volume ($\sim 10^{-9}$cl) there is a marked increase in the scatter of the observed tenth-times. These results have been explained by a mechanism in which gelation is initiated by homogeneous nucleation of polymers in the bulk solution phase, and subsequently, proceeds by heterogeneous nucleation on the surface of existing polymers.

A transient spectrometer has been constructed which is capable of measuring absorption spectra of ordinary solutions in a time of 10 nanoseconds. This spectrometer has been used to monitor the transient recombination of carbon monoxide with hemoglobin after photolysis over the time scale from 10 nanoseconds to 10 milliseconds.

Methods Employed:

Assembly reactions are being studied using a wide variety of physical techniques. The solubility equilibrium between monomer and polymer is studied using sedimentation followed by spectrophotometric analysis of the supernatant solution. This technique permits non-invasive analysis of mixtures of spectrally distinct hemoglobin components, such as oxy-deoxy or carbon monoxy-deoxy mixtures. The composition of the polymer phase, which is spontaneously ordered, is examined by measuring linear dichroism and polarized absorption. These measurements permit quantitative measurements of the polymer phase composition without interference from the protein in the unaligned solution phase.

A variety of light scattering measurements have been employed to study the processes by which polymers are formed. Intensity scattering from a continuous 1.06 micron neodynium YAG laser has been used to study the polymerization and depolymerization of concentrated HbS solutions. These measurements permit detection of polymerized HbS at levels about 10^3 lower than conventional turbidity measurements and hence have provided important information on the time dependence of the onset of polymerization. In addition, turbidity measurements are routinely used to characterize the delay times of samples being studied as mixtures (see above). Intensity scattering is also being used to monitor the kenetics of polymerization in experiments in which gelation is induced by laser photolysis. In this case, scattering of 514 nm light from an argon ion laser is detected at 45° or 135°.

Transient events on the time scale of 10^{-8} to 10^{-1} sec are monitored using a transient spectrometer based on two pulsed Nd:YAG lasers. The development of this spectrometer has been a major effort in the past year. The first laser is used as an excitation source to perturb the sample either by inducing a photochemical change, or by perturbing the solution conditions, using an appropriate light coupled inducer. At present, changes in pH, temperature, pressure and ATP have been coupled to the intense light pulses obtainable from the Nd:YAG laser. Changes in pH and ATP levels, however, require chemical reactions and hence cannot be induced in less than about 10^{-6} sec. A second laser or a continuous monitoring beam is used to measure the time course of the reaction which follows the excitation pulse. In one mode, a pulse of about 10 ns duration from a second Nd:YAG laser is used to excite a solution of a fluorescent dye housed in a thin (250 μ) layer cell. The spontaneous emission from the dye is chosen to overlap the spectral region in which the initial sample and the photoproduct absorb. The emitted light is passed through the sample in two regions, one of which is illuminated by the excitation pulse and the other of which is not. The resulting light is focussed as a spectrograph slit and dispersed. The dispersed image illuminates the face of a vidicon tube in two tracks. The resulting intensity is measured by an optical multichannel analyzer, and after subtracting the dark baseline of the vidicon and taking the log ratio of the resulting intensities, produced a difference spectrum between the excited and unexcited portions of the sample. In this mode the instrument can be used to determine the absorption spectrum of the excited region in about 10 nanoseconds. The second laser pulse is timed

with respect to the first with an absolute accuracy of ± 1 nanosecond, permitting the time evalution of the resulting spectrum to be widely monitored.

The second method used to monitor the reaction is to be measure the time course of the kinetics at a fixed wavelength. This experiment requires a monochromatic source of variable wavelength and a temporal duration which is longer than the kinetic process under investigation. For slow experiments, this requirement is met by DC xenon arc lamps. For fast processes, a source of high intensity is required. Xenon flashlamps having durations of 1 μs to 1 ms produce several hundred times the source brightness of a DC lamp and are used together with existing monochromators to provide single wavelength illumination. A desirable alternative, particularly for resolving small changes in photoselection experiments or very rapid transients, is to take advantage of a CW dye laser as the tuned probe source. The higher photon flux from the dye laser would both improve signal-to-noise and considerably simplify the design of the spectrometer.

For single wavelength kinetic studies, photomultipler tubes are used together with two 200 mHz amplifiers and a dual-channel transient digitizer having 10 ns time resolution. For processing, the data will be rapidly transmitted from the transient digitizer to a small computer, based on an LSI-11 microprocessor which is currently under development by the computer sciences laboratory of DCRT.

Major Findings:

1. Polymerization of Hemoglobin S.

A systematic stu y of the concentration and temperature dependence of the polymerization kinetics has been carried out rising the photolysis technique with light scattering detection described above. There are three major findings from these studies: (1) The apparent concentration dependence of the tenth-time for polymerization decreases by over a factor of 2 as the delay time decreases from 10^{5} seconds to 10^{-3} seconds. (2) The abruptness of the onset of observable polymerization decreases as the delay time decreases. (3) The reproducibility of single experiments decreases markedly as the delay time increases to times greater than 100 sec. The distribution of delay times has been characterized under two sets of conditions, one of which produces an average delay of 15 milliseconds, and the other, an average delay of 80 seconds. The standard deviation of the 15 millisecond delay times was ±5% while the standard deviation of the 80 sec delay times were ±60%, a factor of 12 larger

The measured distribution of delay times and the concentration and temperature dependence has been interpreted with a mechanism based on the nucleation and growth of individual polymers. Two indication processes are required. One in which polymers are nucleated in the bulk solution and a second in which new polymers are formed on the surface of existing polymers. The equations describing this model have been formulated in terms of the thermodynamic properties of the small polymers (nucleii) permitting the kinetic model to be

formulated in terms of only 4 variable parameters. We are currently exploring this parameter space to delimit the larger of acceptable values for these parameters.

The photolysis technique is currently being extended to the study of intracellular polymerization of hemoglobin S. This study has two objectives: (a) a description of the distribution of delay times in the red cell population of patients with sickle hemoglobinopathies and (b) the use of the technique for drug screening. Another project is the investigation of the thermodynamics of gelation in the presence of oxygen. These two projects are described in more detail in the report on hemoglobin S gelation by W. A. Eaton (Z01-AM-29010-08-LCP). These studies are a continuation in collaboration with Dr. H. R. Sunshine of the experiments described in the 76/77 annual report Z01-AM-29016-02-LCP on mixtures of carbonmonoxy- and deoxyhemoglobin S.

2. Transient Absorbance Changes Following Photolysis of Carbonmonoxyhemoglobin.

The initial application of the transient laser spectrometer has been the measurement of transient spectra following the photolysis of carbonmonoxyhemoglobin. These studies were initiated both to develop a standard system with which to measure instrument performance as changes and modifications are made in the apparatus, and to investigate in detail the transient response of hemoglobin prepared by nanosecond photolysis. The studies have showed, in confirmation of the recent experiments of Alpert and collaborators, using transient absorption measurements and of Freidman and collaborators using transient resonance raman experiments, that there is an initial recombination of approximately 50% of the photolyzed CO recombines within about 50 nanoseconds. A second, slower phase is seen in which the absorption spectrum of the remaining deoxy hemes sharpens slightly over a time of about 1 microsecond. These two events are followed by recombination of the unliganded hemes with CO from the solution phase, which takes place between 0.1 and 100 milliseconds.

Significance to Biomedical Research and the Program of the Institute:

The studies of photolysis induced polymerization kinetics provide the only available technique for studying the polymerization reaction as it is actually initiated in vivo, by the removal of ligands on physiological time sides. The fast kinetic studies also show promise as a rapid and convenient screening technique for therapeutic inhibitors of hemoglobin S polymerization.

The physical studies of the polymer phase and polymerization kinetics are aimed at providing a better basic understanding of the physical nature of the polymer phase, and the self-assembly process by which it is formed.

The objective of the transient spectral studies is to provide a detailed picture of the time-evaluation of protein structure. By using photolysis to prepare molecular species which cannot be observed at equilibrium, the pathways by which these species evolve can be determined, providing a new dimension to

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the understanding of the relationship between protein structure and function.

Proposed Course of Project:

Photolysis induced gelation will be extended to the study of intracellular polymerization and to examine gelation kinetics in partially saturated solutions. The present results will be used to delimit the range of parameters for the nucleation-growth model for the kinetics of polymer formation. The photolysis technique will also be used to measure the rates of polymer growth by seeding domains in a predetermined area of a slide.

Continued work on hemoglobin transients will include the time-resolved detection of tryptophase fluorescence, and the investigation of absorption changes in the visible (500-600 nm) spectral region. The fluorescence will be measured by delayed excitation of the sample with 266 nm laser light after photolysis, and will be performed to determine whether the fluorescence quantum yield of aromatic amino acids can be used to monitor changes in tertiary or quaternary structure.

The transient spectrometer will be generalized to permit polarized excitation and detection. In addition, transient microspectrophotometry will be explored to determine whether we can measure transient events after photolytic excitation on samples as concentrated (0.69 g/cm^2) as hemoglobin single crystals.

Publications:

Hofrichter, J., Sunshine, H.R., Ferrone, F.A. and Eaton, W.A.: Oxygen binding and the gelation of sickle cell hemoglobin. In *Proceedings on the Symposium on the Molecular Basis of Mutant Hemoglobin Dysfunction*, P. B. Sigler (Ed.), Elsevier, North Holland (in press).

Hofrichter, J.: Molecular Description of Sickle Hemoglobin Gelation. In *The Combined Clinical Staff Conference on Scientific Basis of New Therapeutic Approaches in Sickle Cell Anemia*, Annals of Internal Medicine (in press).

Ferrone, F.A., Hofrichter, J., Sunshine, H.R. and Eaton, W.A.: Kinetic studies on photolysis induced gelation of sickle cell hemoglobin suggest a new mechanism. In *Biophysical Discussion on Proteins and Nucleoproteins: Structure, Dynamics and Assembly*, Biophysical Journal (in press).

Sunshine, H.R., Hofrichter, J., Ferrone, F.A. and Eaton, W.A.: Oxygen binding by sickle hemoglobin polymers. In *Interaction of Iron With Progein in Oxygen and Electron Transport* (in press).

Eaton, W.A. and Hofrichter, J.: Linear dichroism of hemoglobin methods in enzymology (in press).

REPORTED IN PRESS - 1979

Sunshine, H.R., Hofrichter, J. and Eaton, W.A.: Gelation of sickle hemoglobin in mixtures with normal adult and fetal hemoglobins. J. Mol. Biol. 133: 435-467 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29017-01-LCP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Spectroscopic investigation of membrane lipids and models

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Ralph G. Adams	Research Physicist	A LCP
OTHER:	Ira W. Levin	Research Chemist	A LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Infrared and Raman spectroscopic studies have been used to characterize conformational changes in the gel and liquid crystalline forms of simple model membrane systems.

Project Description

Studies of dipalmitoyl phosphatidylcholine have given insight into the role of water and cholesterol in lipid membranes (see Levin).

Didodecyl dimethyl ammonium bromide (DDAB) has been proposed as a model membrane former and has the advantage of a very small headgroup with no carbonyls. We have used Raman and infrared studies of DDAB to show that cholesterol's ability to disorder lipid bilayers is controlled by the carbonyl headgroups to some extent. It is also evident that DDAB can be used to simplify model systems which will aid in investigating membrane activities. The gel-liquid crystalline behavior of DDAB has been characterized by temperature profiles determined from changes in the vibrational infrared and Raman spectra of both multilamellar and vesicle systems.

Future Work

Further questions about cholesterol's function in membranes will be investigated.

We are attempting to find a spectroscopic probe to differentiate between inner and outer layers of bilayer structures using selected infrared and Raman vibrational transitions.

Publications

Bush, S. F., Adams, R. G. and Levin, I. W.: Structural reorganizations in lipid bilayer systems: effect of hydration and sterol addition on the Raman spectra of dipalmitoyl phosphatidylcholine multilayers. Biochemistry. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29018-01-LCP

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
The development of radiocryptate assisted imaging methods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Otto A. Gansow IPA A LCP

COOPERATING UNITS (if any)
Mark H. Rotman (CC-NM)
John Goble (ORS-S-RS)
Robert M. Kessler (CC-NM)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Synthesis of a potential new class of radiotracers, metal cryptate complexes of trivalent metal ion radioisotopes, has been effected. This project will explore and develop in vivo and in vitro applications of these new materials. In particular, it now seems clear that positron axial tomography (PAT) techniques will soon provide better images of tissues, blood vessels and organs in vivo than now available from gamma camera pictures. Several isotopes which form chemically stable trivalent ions could be most useful as positron sources. Recent studies indicate that ^{68}Ga(III) ion is likely to be the most important. Its chemical and physical properties endow it with the potential for supplanting ^{99m}Tc as the most medically useful radioisotope. The gallium cryptates and the metal cryptate complexes of several lanthanide and actinide ions as well as those of In(III) were first explored during this past year.

Experiments completed to date include the development of safe techniques for encryptating radioisotopes, the synthesis of Ce(III) cryptates, the first in vivo injections of that trivalent cryptate, and the acquisition of γ-camera images to explore in vivo distribution of metal cryptates.

Rationale for the Project

The premise upon which this project is based is that certain isotopes which form trivalent metal ion possess physical and chemical properties which will make them useful in nuclear medicine. The physical properties of the isotopes (half-life, β^+ emission, γ energies, etc.) must be such as to be appropriate for radiation detection by a gamma camera, or better, by a positron axial tomograph. The chemical nature of the trivalent complexes employed must be such as to insure their chemical integrity so that when incorporated in a radiopharmaceutical, or other biological material, stability and inertness are ensured.

The lanthanide, actinide and Group IIIA elements have members which possess the desired physical and chemical properties. Synthesis and characterization of representative metal cryptate complexes has been effected at Michigan State University. Stability and inertness have been explored and found adequate for the uses intended.

Extensive prior work in nuclear medicine shows that when these ions are placed in vivo as weak complexes or ions free in solution, they are rapidly and, in general, non-specifically distributed. In certain cases, for example, Ga(III) citrate, they become fortuitously concentrated in small amounts in places that render them useful, e.g. the brain. This frequently requires the use of large doses of radiation and extended waiting times for clearance of background radiation. We hope that by preparing inert complexes which can be specifically (covalently) attached to molecules with known biological specificity, we can take advantage of that specificity to do efficient in vivo labelling and imaging, with attendant improvement in resolution and image to background ratio. A methodology for the project is provided below.

Methodology for the Project

Certain necessary steps required for the development of metal cryptate tracers can be outlined:

a) Synthetic procedures used to prepare "cold" materials must be modified to prepare encryptated radioisotopes.

b) Initial in vivo studies must be performed to test toxicity and tissue distribution of cryptates initially free in solution.

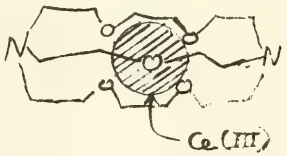
c) Bifunctional cryptates suitable for attachment to pharmaceuticals, antibodies or immunogens must be prepared.

d) Cryptate labelled biologicals must then be prepared and their in vivo activity and distribution explored for utility in nuclear medicine.

Progress to date include the completion of step a and partial completion of steps b and c, as outlined below.

Results

Procedures initially employed to make trivalent metal cryptates called for preparations in oxygen and water free environments and for the rigorous prepurification and drying of all chemicals employed. After some exploration, it soon became evident that one could not expect radiopharmacy personnel to easily adapt to such methods. Modifications were in order. A procedure was developed whereby dehydration of a suitable solvents was effected by reaction of contained water with triethylorthoformate (TEOF). Similarly, it was discovered that our initial ions of choice, the early trivalent lanthanides, could be dehydrated in situ by reaction with TEOF in CH_3CN. Rapid combination of these two solutions in air led to cryptate formation. The technology is such as to be easily used by technician rather than professional personnel. The cryptate prepared and employed in our initial studies was the $[Ce(2:2:1)]Cl_3$ pictured below.



It is the unique cage-like structure of the ligand that causes the chemical inertness of the molecule. In effect, the metal ion is restrained inside the cage.

The rabbit was used for all in vivo tests to date. It was observed that when large doses of the lanthanide ion Ce(III) free in normal saline solution were given intravenously in the ear, convulsions and hemorraging soon followed. The experiment was terminated soon thereafter for safety reasons. However, after encryptation, doses ten times in excess (and perhaps greater) can be given to the rabbit with no apparent ill effects. The rabbits lived contently until termination of following experiments, usually 8-12 hours after injection. These experiments are important in that they show the cryptates not to be initially toxic even in large (>100 mg/kilo) doses.

Gamma camera pictures obtained over 8-12 hours after injection showed the following:

a) Rapid uptake of isotope in the brain, with subsequent loss of radioactive material over the course of 1/2 hour.

b) Rapid vascular distribution of the cryptate.

c) Less rapid but extensive uptake of the cryptate in the liver and spleen.

d) Slow uptake in the kidneys and bladder.

All experiments performed used ∿ 50 mg of $CeCl_3$ carrier in addition to ca. 1.0 mCi of ^{141}Ce. Carrier free experiments will be performed soon.

The loss of material from organs in time is undesirable and could be caused by cryptate dissociation. We therefore plan to repeat this work with the more stable cryptates of the Group IIIA elements. There is some hope that if suitable blocking procedures and isotope retention times are obtained, a useful positron brain imaging material will have been found.

Work being performed at Michigan State this past year has resulted in two bifunctional cryptand ligands suitable for attaching the entire metal complex as a radiotracer. Briefly, the amino benzo group ($H_2N-C_6H_4-$) and the amino-catechol $H_2N-C_6H_3O_2-$ groups have been incorporated into the cryptand ligand structure to provide sites for covalent attachment. Bifunctional, attached cryptates should be specific, stable labels. This potential use will be extensively explored next year.

Planned Research

First, we plan to develop procedures by which the Ga(III) cryptates may be prepared in a radiopharmacy environment. We plan to preform in vivo studies of tissue, blood vessel and organ uptake of these materials. Positron imaging methods will be employed.

Secondly, bifunctional radiocryptates of the lanthanide and Group IIIA metals will be prepared and covalently attached to pharmaceuticals and proteins. Synthesis of thyroxine and digoxin attached cryptates is nearing completion at Michigan State. Research at NIH will concentrate on attachment of cryptates initially to blood proteins such as albumin. This will allow direct comparison between cryptate labelling and more conventional methods, such as radioiodination. Next, immunoproteins will be labelled and attempts made to observe their in vivo reaction at antigen binding sites, with the hope that biological specificity will be maintained. In vivo studies of the cryptate labelled radiopharmaceuticals will also be preformed. The specific goal in mind is to find chemicals and biologicals that are sufficiently specific in site location and sufficiently stable to be useful for imaging and diagnosis in nuclear medicine.

ANNUAL REPORT
LABORATORY OF PHYSICAL BIOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Muscle Contraction

X-ray diffraction. Considerable progress was made in the effort to obtain structural information from simplified muscle preparations under chemically and mechanically controlled conditions. Using "skinned" rabbit psoas fibers, stable X-ray diffraction patterns were obtained under relaxed, calcium-activated, and rigor conditions. At 10°C, the intensity ratio of the two main equatorial reflections, I_{11}/I_{10}, increased more than three-fold as the degree of activation by calcium was increased to the maximum value. The intensity ratio of partially activated skinned rabbit fibers, as a function of relative force, was close to that found previously with intact frog muscles activated by caffeine. The intensity ratio increased another factor of two when ATP was removed from the bathing solution and the fibers passed into the rigor state. These results are consistent with the idea that the change in intensity ratio upon activation is caused by the formation of cross bridges between the myosin-containing and actin-containing myofilaments. The difference between the intensity ratio in the calcium-activated and the rigor state indicates that cross bridges develop force by passing through a cycle containing several states and that the this cycle is interrupted when ATP is removed from the system. (Matsubara, Yu)

In fibers fully activated by calcium the intensity ratio and steady force were almost the same at high (5 mM) and low (0.02 mM) concentrations of MgATP. This is interesting because the contraction speed decreases considerably when the concentration of MgATP is lowered from 5 to 0.02 mM. The results suggest that contraction speed may be affected by the rate at which cross bridges are "broken" by MgATP when the bridges are strained due to relative motion of the myosin-containing and actin-containing myofilaments. (Matsubara)

Small bundles of glycerinated rabbit psoas fibers were placed in an ATP-free salt solution and subjected to a small step increase in length (30 Å/half sarcomere, the displacement produced by the cross bridge cycle in shortening muscle fibers). Under these conditions the cross bridge cycle is interrupted and all the myosin heads remain attached to actin. The X-ray diffraction pattern, axial force, and sarcomere length were monitored using electronic position sensitive detectors. Following stretch the lattice spacing remained constant and the average intensity ratio I_{10}/I_{11} decreased 40%. I_{10} decreased and I_{11} increased relative to their initial values. Therefore straining a rigor cross bridge moves it closer to the thin filament. This is structural evidence that physiological force development and cross-bridge configuration are closely coupled. (Naylor)

Synchrotron radiation. Diffraction experiments with muscle cells were made with the intense X-ray source at the Stanford Synchrotron Radiation Laboratory during a run dedicated to the production of X-rays. The experiments were designed to examine the effect of physiological activation on the equatorial reflections that lie beyond the generally studied strong [1,0] and [1,1] reflections. These high order reflections ([2,0], [2,1] [3,0], [2,2], and [3,1]) are just measurable in relaxed frog muscle fibers using conventional laboratory X-ray sources.

It was found that $I_{2,0}$, $I_{2,1}$, and $I_{3,0}$ become much weaker when the muscle is activated; this contrasts sharply with $I_{1,1}$, which increases several fold upon activation. The [2,2] and [3,1] reflections, which form a doublet, were almost the same in the relaxed and activated states. It was also observed that equatorial patterns of muscle in contraction and in rigor were similar in that the higher orders beyond [1,1] were considerably weaker than [1,1], indicating that cross bridge attachment is similar in both states. These observations will be analyzed further by detailed modeling of the myofilament lattice as a function of cross bridge configuration. (Yu, Naylor, Podolsky)

Rate limiting step in the cross bridge cycle. We have found that the steady contraction velocity of skinned muscle fibers is not affected by parameters (ionic strength, nucleotide concentration, etc.) that change the transient response of the fiber. This "decoupling" of the steady motion from the transient response cannot be explained by a two-state cross bridge cycle where the myosin moiety that projects from the myosin-containing filament is either ready to become, or already part of, a cross bridge.

Eisenberg, Hill, and Chen recently put forward a more general four-state model in which the rate limiting step for moderately loaded contraction is a slow transition of the myosin molecule from a non-reactive to a reactive state. However, if the reaction sites on the actin containing filament are 360 A apart (the pitch of the actin helix), as was assumed, this model does not decouple the steady motion from the transient response either. It appears that the cross bridge attachment sites on the actin-containing filament may be considerably more closely spaced than the pitch of the actin helix. (Yu, Podolsky)

State of the myosin molecules in relaxed muscle. While active muscle is well described in terms of cross bridges being responsible for the elastic behavior of the system, resting muscle is more complicated in that it exhibits viscous as well as elastic behavior. It has been shown that the mechanical response of resting muscle can be separated into two distinct phases. The viscous phase has a temperature coefficient (Q_{10}) of 1.4, increases with sarcomere length, but does not change much shortly after stimulation. In distinction, the elastic portion of the response has a Q_{10} of < 1.1, also increases with sarcomere length but increases dramati-

cally upon stimulation. The elastic phase was found to be most likely due to passive structures at rest and to activated cross bridges shortly after stimulation. Computations raised the interesting possibility that the viscous behavior of resting muscle might be due to rapidly attaching and detaching cross bridges. (Schoenberg)

The activation mechanism. Analysis of stimulated ^{45}Ca efflux in skinned fibers was extended. Caffeine both stimulates efflux directly and inhibits active Ca transport into the sarcoplasmic reticulum. The efflux stimulation consists of a very small independent component tightly coupled in series to a large Ca^{2+}-dependent component, which is not strongly inhibited by free magnesium unless free calcium is very low. The Ca^{2+}-gated channel shown to mediate most or all of the stimulated ^{45}Ca efflux in skinned fibers thus is likely to function in the intact fiber. Simultaneous inhibition of active calcium transport during stimulation suggests a possible link between the Ca^{2+}-gated channel and the Ca pump ionophore; inhibition following stimulation by ionic depolarization was suggested previously by analysis of ^{45}Ca flux, and the hypothesis is now supported by recent published work on electrically stimulated "intact" fibers. This adaptive inhibition could result from a common element in the pathways. (Stephenson)

Two review articles were prepared dealing with activation of fast skeletal muscle. One of these coordinated physiological, biochemical and morphological observations related to both membrane control of intracellular calcium movement and calcium control of the contractile system. The second, which was more specialized, was concerned with magnesium ion effects on regulation of skinned fibers. (Stephenson)

Cell Membranes

Phase transitions in lipid films and cell membranes. The existence of a crystalline lipid state in cell membranes has been established by a number of independent studies. Since it is generally assumed that membrane lipids are arranged only as a single bimolecular film, it has been deduced that this membrane bilayer can exist in a crystalline state. However, it has now been demonstrated that single bilayers can be prepared which do not form crystalline states. Crystalline phospholipid states have been reported for multibilayer systems; no direct measurements of crystal states in single bilayers have ever been reported. Therefore it is proposed that the observation of crystalline states in lipid membranes is due to the presence of non-unibilayer states. This proposal is in agreement with recent freeze-fracture evidence of lipid membranes. (Gershfeld)

A quantitative mass spectroscopic method for disaturated lecithins has been developed. This is the first quantitative analysis of triglycerides achieved with this technique, and supplements the present gas

chromatographic approach. This procedure will make it easier to analyze plasma for disaturated lecithins and to see whether the levels of these substances are associated with atherosclerosis. (Gershfeld)

Red cell membrane and cholesterol. Previous work has shown that the rate determining step in cholesterol transport from plasma lipoproteins to human erythrocytes may be the diffusion of cholesterol in the cell membrane away from a lipoprotein binding site. Consistent with this, the cholesterol transfer rates have now been found to be independent of the solution ionic strength, a factor which would be expected to alter the rate of collisions of lipoprotein molecules with the cell surface. In addition, the number of binding sites for low density lipoprotein computed on the basis of membrane-limited diffusion was found to be 300. This is in essential agreement with the number found by others from direct binding studies. (Gottlieb)

The Nature of Macromolecular Damage by Ionizing Radiation

Analysis of the effects of ionizing radiation on matter has been based on classical experiments with air and diatomic gas molecules. The phenomena and mechanisms are found to hold for condensed matter also. In addition, there are effects which are only observed in irradiation of macromolecules. These effects are common to proteins, nucleic acids and synthetic plastics. They include energy transfer along polymer chains, lack of energy transfer between chains, and a strong temperature dependence of radiation sensitivity. Explanation for these observations depends on the nature of macromolecular structure and the coupling of adjacent atoms in the polymeric backbone. (Kempner)

Morphogenesis

Insect metamorphosis. Calliphorin is the major storage protein in insects. Contrary to earlier reports, calliphorin hexamer appears to consist of nine rather than six individual monomeric species, which complicates the isolation problem. As a last resort, conditions have been worked out for separating the hexameric species by preparative isotachophoresis, a new procedure than operates on differences in protein isoelectric point. Physico-chemical characterization of calliphorin has also been undertaken. Extensive use of computer modeling makes it possible to surmise which amino acid residues are on the protein surface, and what type of forces are involved in oligomer formation. These conclusions are in good agreement with those derived from chemical modification experiments. (Levenbook, Bauer, Zomer)

Red cell sickling. In many self-assembly processes (e.g. tubulin, mitotic spindle, actin, flagellin, TMV coat protein) hydrostatic pressure inhibits polymer formation. A similar effect has been observed in sickle hemoglobin (HbS) gelation. The temperature-induced gelation of purified deoxyHbS in a jacketed high pressure optical cell has been monitored by

following the turbidity at 820 nm. As the hydrostatic pressure increases from 1 to 700 atm the characteristic delay time increases, the rate of absorbance change at 820 nm upon gelation decreases, and the extent of polymer formation decreases. The equilibrium volume change of the reaction cannot be accurately obtained with this technique. However, when the change in gelation kinetics with pressure is incorporated into the theory of rate processes, the data yield a positive volume change of activation of approximately 100 cc/mole--strongly suggestive that in at least one step of the polymerization process, the partial specific volume of HbS, increases. (Adams, Murayama)

Bioluminescence

Field and laboratory evidence indicate that rover synchrony involves direct male-male sequential excitation, resulting in wave-like spread of luminescence through the congregation. The male-male triggering depends on a male-sensitivity "window" during the male's pacemaker cycle in addition to a window during which stimulation elicits responses appropriate to courtship of a female. To account for the difference between rover behavior and habitual synchronizer behavior, it is postulated that in rovers an exogenous signal causes an immediate abortion of the timing cycle and dispatch of the terminal neuromotor message to the firefly's lantern whereas in habitual synchronizers the signal resets the pacemaker. (Buck)

From study of photocyle granule ultrastructure in Photinus pyralis several types or stages have been recognized. Photocyle granules of Photuris versicolor have been subjected to immunochemical testing using rabbit and rabbit-goat antisera to crystalline Photinus pyralis luciferase. Antibody conjugated with either peroxidase or ferritin was found to localize heavily in the photocyle granules. (Neuwirth)

High Resolution Electron Microscopy

An extensive laboratory renovation was completed which allowed (1) installation of a Philips EM 400 electron microscope with image instensifier, geometric stage, and radiation reduction kit, and (2) construction of an optical diffractometer. In addition, computer software for image processing has been extended by developing programs to transfer digital images from the analysis programs implemented on the IBM 370 computer to the graphics hardware and analysis system . The major advance represented by these developments is a substantial improvement in the quality and variety of photographic output of electron microscope images after digital image processing. (Steven, Trus (NIDR))

Electron microscopy of immunoglobin crystals. Using digital methods, electron microscope images of negatively stained microcrystals of a human

myeloma immunoglobulin have been filtered. These images were then compared to "ideal" micrographs, derived computationally from the crystal structure. From a priori considerations, potentially severe perturbations of molecular structure might be caused by dehydration, by fixation, by electron irradiation, by chemical reaction with the stain, by multiple electron scattering, or by microscope aberrations. The central conclusion of this study is that, to an effective resolution of about 2.0 nm, the electron microscope images convey a remarkably accurate representation of the molecule. This approach to crystal structure with electron microscopy can be applied to crystals which are too small, or otherwise unsuitable for analysis by conventional methods of X-ray crystallography. (Steven, Navia (LMB))

Ultrastructure of beetroot yellow necrotic virus (BYNV). Plant viruses represent important, experimentally accessible, systems for studying the assembly of nucleo-protein particles. A combination of electron microscopy, optical diffraction and image processing has been used to determine the structure of BYNV. Our results on the structure of this filamentous virus particle show that the packing of protein subunits is based on a single-stranded right-handed helix which returns to its angular origin after four helical turns. (Steven, Wurtz (University of Basel))

Paracrystalline tetragonal particle arrays in the human erythrocyte. Regular patches of tetragonally arranged particles have been observed in erythrocyte membranes. There appear to be several morphologically distinct classes of patch. The protein(s) which constitute these particles have not been identified yet, although SDS-polyacrylamide gel electrophoresis of the membranes on incubation show protein patterns characteristic of erythrocyte ghosts, modified by a moderate degree of limited proteolysis and partial release of the cytoskeletal proteins, spectrin and actin. (Gottlieb, Steven)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27000-18 LPB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Mechanism of Muscular Contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Podolsky	Chief, LPB	LPB NIAMDD
OTHER:	L. C. Yu	Research Physicist	LPB NIAMDD
	E. W. Stephenson	Expert	LPB NIAMDD
	G. R. S. Naylor	Visiting Fellow	LPB NIAMDD
	S. Matsubara	Visiting Associate	LPB NIAMDD
	T. Arata	Visiting Fellow	LPB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Cellular Physics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7	5	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Muscle cells are activated when calcium is released from the sarcoplasmic reticulum into the myofilament space. The processes involved in this intracellular calcium movement have been studied by measuring isometric force and ^{45}Ca fluxes in mechanically skinned fibers from frog muscle. The structural changes associated with the transition of muscle cells from one physiological state to another have been examined by X-ray diffraction techniques, using radiation from conventional rotating anode generators and synchrotrons in conjunction with electronic, position sensitive, X-ray detectors. The rate constants associated with the turnover of actomyosin cross-bridges have been studied by analyzing the contraction kinetics of skinned muscle fibers under controlled chemical conditions.

PHS-6040

Project Description:

Objectives:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.
2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.
3. Measurement of ionic fluxes across the internal membranes of skinned muscle fibers under various conditions.

Major Findings:

1. The intense X-ray source at Stanford Synchrotron Radiation Laboratory was used to record the high order equatorial intensities in relaxed and in electrically activated frog muscle. Upon activation the orders [2,0], [2,1], and [3,0] became very weak. This contrasted markedly with the [1,1] reflection which increased several fold. The doublet [2,2] and [3,1] was almost unchanged. It was also observed that equatorial patterns of muscle in contraction and in rigor were similar in that the higher orders beyond [1,1] were considerably weaker than [1,1], indicating that cross-bridge attachment is similar in both states.

2. The intensities of the two strong equatorial reflections, I_{10} and I_{11}, were measured in small bundles of chemically "skinned" rabbit psoas fibers using a laboratory X-ray generator. The change in intensity ratio I_{10}/I_{11} during maximum activation by calcium was less than that seen in the rigor state produced by removal of ATP. In partially activated fibers the intensity ratio was a non-linear function of relative force. The relation between intensity ratio and relative force was close to that found previously in intact frog muscle fibers where the degree of activation was controlled indirectly by caffeine.

3. In rigor muscle fibers the intensity ratio I_{10}/I_{11} decreased 40% when the sarcomere was strained 30 A/half sarcomere. This indicates that strain causes cross bridges to move closer to the actin-containing myofilament and that the equatorial diffraction pattern is sensitive to the configuration as well as the number of cross bridges.

4. Caffeine stimulates the movement of calcium from the sarcoplasmic reticulum into the myofilament space by means of a very small

independent component which is tightly coupled in series to a large Ca^{2+} dependent component. The Ca^{2+} gated channel probably functions under conditions present in the intact fiber.

5. The steady motion and the transient response of skinned muscle fibers can be decoupled experimentally. This behavior was not apparent in calculations made with the four-state cross bridge model put forward by Eisenberg, Hill, and Chen. The results suggest the cross bridge attachment sites on the actin-containing filament may be considerably more closely spaced than 360 A, the pitch of the actin helix.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the molecular mechanism of muscular contraction together with the chemistry of the activation process, can be useful in the rational handling of neuromuscular and cardiovascular disease.

Proposed Course of Project:

1. The influence of various agents (pCa, pH, ionic strength, metal ions, ATP) on the ability of muscle fibers to develop force and to shorten will be examined directly in skinned fiber segments.
2. The influence of these same agents on myofilament spacing and the cross bridge configuration will be examined in skinned fibers by X-ray diffraction.
3. The effect of motion on the number of cross bridges in intact, electrically-stimulated muscle fibers will be studied by X-ray diffraction.
4. Movement of calcium and magnesium between the myofilament space and the sarcoplasmic reticulum will be studied by tracer methods.

Publications:

Podolsky, R. J. and Tawada, K.: A rate limiting step in muscle contraction. Oji Symposium on Regulatory Mechanism of Muscle Contraction, Japan Scientific Societies Press, Tokyo (in press).

Stephenson, E. W.: Activation of fast skeletal muscle: contributions of studies on skinned muscle fibers. Am. J. Physiol. (in press).

Stephenson, E. W.: Magnesium effects on activation of skinned fibers from striated muscle. Fed. Proc. (in press).

Reported in Press, 1979

Yu, Leepo C., Hartt, Jacqueline E., and Podolsky, Richard J.: Equatorial X-ray intensities and isometric force levels in frog sartorius muscle. J. Mol. Biol. 132: 53-67, 1979.

Podolsky, Richard J.: The rate limiting step in muscle contraction. Basic Res. Cardiol. 75: 34-39, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 27001-06 LPB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Contractility and Excitability of Skeletal and Cardiac Muscle

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Mark Schoenberg Medical Officer LPB NIAMDD

COOPERATING UNITS (if any)
Dr. Jay B. Wells, Medical Neurology Branch, NINCDS

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Cellular Physics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanical response of resting and activated muscles to imposed length changes has been measured. The response has a viscous and an elastic component. The latter is due to passive structures in the resting muscle and activated cross bridges in the stimulated muscle. The origin of the former remains uncertain.

PHS-6040

Project Description:

Goal: To understand the molecular and structural basis of muscular contraction.

Methods and Major Findings:

Evidence suggests that in muscle, under certain conditions, myosin cross-bridges attach cyclically to the actin filament. They are believed responsible for the stiffness of activated muscle. Work in FY'79 was directed towards understanding to what extent the behavior of resting (non-stimulated) muscle could be explained by cross-bridges and also, how the behavior of the resting muscle changes upon activation.

1) Experimentally it was shown that the mechanical response of resting muscle had two distinctly separate phases: phase I exhibited viscous behavior, and phase II was dominated by the stiffness of the muscle. The temperature coefficient (Q_{10}) for phase I was 1.4 and for phase II was less than 1.1.

2) Computations were made which suggested that if cross-bridges attach and detach as rapidly as recent biochemical evidence suggests, then in the resting muscle they may be weakly attached to the actin filament and be responsible for of the resting "viscosity."

3) The stiffness of the muscle, determined from phase II was shown to be likely due to passive structures in the resting muscle. Upon stimulation, this portion of the response increases, presumably because of the appearance of activated cross-bridges.

4) The viscous portion of the response was found to remain the same (or perhaps slightly decrease) initially upon activation. It increased with increasing muscle sarcomere lenth. Although theoretical calculations (see 2) raise the interesting possibility that this portion of the response might be due to attached cross-bridges, at present its etiology remains unknown.

5) If the viscosity of resting muscle remains the same throughout activation, its effect upon mechanical wave propogation in an activated muscle (see FY 76-78) would be $< 10\%$.

Significance to Biomedical Research and the Program of the Institute:

Experiments on the mechanism of muscular contraction are especially significant in that they contribute to a sound understanding of how normal muscle works (which is vital to the study of diseased muscle) and also because many of the same mechanisms responsible for muscle contraction appear to be common to virtually all cells.

Future Course:

Experiments are planned which should help elucidate the etiology of phase I in the resting muscle. Characterizing the effect of ionic strength and interfilament separation upon the mechanical response to stretch should prove useful.

Publications:

Schoenberg, Mark: Geometrical factors influencing muscle force development. I. The effect of filament spacing upon axial forces. Biophys. J. 30: 51-68, 1980.

Schoenberg, Mark: Geometrical factors influencing muscle force development. II. Radial forces. Biophys. J. 30: 69-78, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-27002-17 LPB

PERIOD COVERED: October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Sense of Rhythm

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John B. Buck	Research Physiologist	LPB-NIAMDD
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	Dr. Maria Neuwirth	Visiting Fellow	LPB-NIAMDD

COOPERATING UNITS (if any)

Mr. George Atta, DCRT-NIH

LAB/BRANCH: Laboratory of Physical Biology

SECTION: Comparative Physiology Section

INSTITUTE AND LOCATION: NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A manuscript on flash synchronization in roving-type fireflies has been completed. This paper compares the rare and usually loose concerted flashing among most species of North America and Europe, which court via exchange of timed signals between male and female, and the habitual and precise mass synchrony typical of many gregarious species of the tropical Orient.

Luciferase has been localized to the photocyte granules, via immunochemical E.M.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The ultimate objective of understanding the control of light emission is being attacked at two levels, (1) the neuroeffector mechanism as deduced from temporal light-emission patterns during free run and exogenously triggered flashing, and (2) the ultrastructural distribution and changes in the chemiluminescent reactants.

Major Findings:

Field and laboratory evidence indicate that rover synchrony involves direct male-male sequential excitation, resulting in wave-like spread of luminescence through the congregation. The male-male triggering depends on a male-sensitivity "window" during the male's pacemaker cycle in addition to a window during which stimulation elicits responses appropriate to courtship of a female. In Photinus pyralis the early window (female-tuned) is about 1 s long and occurs between 1.5 and 2.5 s after the male's flash. The male-tuned window is also about 1 s long and occurs between 4 and 5 s after the male's flash. To account for the difference between rover behavior and habitual synchronizer behavior I have postulated that in rovers an exogenous signal causes an immediate abortion of the timing cycle and dispatch of the terminal neuromotor message to the firefly's lantern (minimum male-male latency 0.3 to 0.4 s) whereas in habitual Oriental synchronizers the signal resets the pacemaker (latency of one full free run period). In a capacitative model, rover entrainment can be diagrammed as an immediate rise of excitation to the triggering level (termination) whereas gregarious entrainment involves an immediate fall of excitation to the basal level, tantamount to re-initiating the endogenous timing cycle. (Buck)

Movie records of congregational flashing in an American roving-type firefly were analysed statistically and shown not to be explicable by chance. (Atta)

From study of photocyte granule ultrastructure in Photinus pyralis several types or stages have been recognized one of which has interesting associations of large intra-granule crystals with arrays of minute tubules. Photocyte granules of Photuris versicolor have been subjected to immunochemical testing using rabbit and rabbit-goat antisera to crystalline Photinus pyralis luciferase (Luciferase courtesy Dr. Marlene Deluca, Univ. of California). Antibody conjugated with either peroxidase or ferritin was found to localize heavily in the photocyte granules. (Neuwirth)

Project No. Z01 AM-27002-17 LPB

Significance to NIAMDD Research:

Insofar as the Institute supports attempts to broaden our understanding of basic biological processes it is hoped that our work is appropriate.

Proposed Course of Project:

At least two more studies of pacemaker control of flashing in tropical oriental fireflies need to be written up (data in hand largely analysed). Overall objective is monographic summary of firefly physiology.

Publications:

Buck, J. and Buck, E.: Flash synchronization as a tool and enabler in firefly courtship competition. Amer. Naturalist. In press, (Oct., 1980).

REPORTED IN PRESS -1979

Ballantyne, L. A. and Buck, E.: Taxonomy and behavior of Luciola (Luciola) aphrogeneia, a new surf firefly from Papua New Guinea. Trans. Amer. Ent. Soc. 105: 117-137, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27003-11 LPB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Dynamic Properties of Cell Membranes and Related Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Norman L. Gershfeld Research Chemist LPB NIAMDD

COOPERATING UNITS (if any)
S. Razin, Department of Clinical Microbiology, Hadassah Medical School Jerusalem, Israel

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Comparative Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major membrane phospholipids--lecithin, sphingomyelin, and phosphatidyl ethanolamine--have been shown to form spontaneously single bilayers at the water surface. Mixtures of these lipids will also form surface bilayers, but at temperatures which are intermediate between the temperatures at which the pure compounds form surface bilayers. The surface bilayers form only when in the presence of bulk lipid. The process of formation of the surface bilayers parallels the formation of membrane bilayers in that for both systems the saturated fatty acid composition increases when temperatures are elevated. The significance of this correlation as a possible mechanism for membrane biogenesis is being tested by studying the growth characteristics of the temperature sensitive bacillus stearothermophilus.

PHS-6040

Project Description:

Major Findings:

I. Phase transitions in lipid films and cell membranes.

Phase transitions in cell membranes have been examined by a number of investigators, and the major one has been identified as the gel-liquid crystal transition of the phospholipids. However, it is generally believed that this transition occurs in a single bimolecular state in the membrane. In our own studies we have attempted to demonstrate that single lipid bilayers would not form the gel (crystalline) state, and presented results that phospholipids form single bilayers only at temperatures where the gel state does not exist (see Annual Report, 1979). Counter arguments have been presented by others in which it has been claimed that monomolecular films of lecithins undergo a gel-liquid crystal transition, indicating that even monomolecular films of lecithins will form the crystalline (gel) state. However, we have also been able to demonstrate that these monolayer reports are in error, because the films which have been purported to be in the monomolecular crystal (gel) form do not exist as such, but are metastable, multimolecular, and are a consequence of the use of a solvent to form the film. We believe that the presence of gel state lipids in membranes signifies the existence of at least a portion of the membranes lipids in a bulk state--perhaps 2-3 bilayers thick. In support of this hypothesis, recent freeze fracture results with pure lipid systems indicate that some non-unibilayer state (previously attributed to membrane protein) can exist in membranes (DeKruiff et al.)

II. Disaturated lecithin analysis in plasma and atherosclerosis.

We are continuing our studies of the relation between disaturated lecithin titer in plasma and atherosclerosis. Our main tool has been the gas chromatograph, and this has been supplemented with an automatic sampling device which has allowed us to increase the number of samples analyzed per week from 2 to about 20. These studies are continuing, with further collaboration from the NHLBI which will provide blood samples from all patients who have been diagnosed to have atherosclerosis.

Further efforts to develop another analytical technique for quantitation of plasma disaturated lecithin levels has been achieved with the mass spectrometer. At present this technique is still cumbersome, and does not lend itself to automatic sampling. However it provides an alternative method of analysis to the gas chromatograph, and therefore will reinforce our confidence in, and reliability of the gas chromatographic method.

Future Course of Study:

We are presently examining the dynamic and equilibrium properties of surface bilayers and their possible significance to membrane structure. A technique for identifying the temperature of surface bilayer formation of any phospholipid mixture has been developed. We shall also continue examining disaturated lecithin content of plasma from patients with various diseases, in which there is a high incidence of atherosclerosis, e.g. diabetes and hypocholesterolemia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 27004-10 LPB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Physical Chemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Melvin H. Gottlieb Research Chemist LPB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Comparative Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Work on the mechanism of cholesterol transport from plasma lipoproteins to human erythrocytes was brought to a conclusion with the finding that transport rates were found to be independent of ionic strength of the plasma. This is in accord with our earlier conclusion that the rate determining step is the diffusion of cholesterol in the erythrocyte membrane. Further, direct determination of lipoprotein binding sites on erythrocytes by other workers were in agreement with those predicted on the basis of the membrane diffusion limit. A study was begun of the effects of species type and modifications of mammalian erythrocyte membranes on the rates of transmigration of phospholipids between the inner and outer halves of the membrane phospholipid bilayer. This work should result in molecular level information on the structure of cell membranes.

PHS-6040

Project Description:

Work of the past year can be divided into three sections, the first of which consisted of winding up earlier work on the mechanism of cholesterol transport from plasma lipoproteins to human erythrocytes. This earlier work had shown that the rates of this transport were consistent with the rate determining step being the diffusion of cholesterol in the cell membrane away from a lipoprotein binding site. Consistent with this, the cholesterol transfer rates have now been found to be independent of the solution ionic strength, a factor which would be expected to alter the rate of collisions of lipoprotein molecules with the cell surface. Further, other workers (Hui, D. Y., Noel, J. G., and Harmony, J.A.K. BBA, In press) have found from direct binding studies that human erythrocytes have about 200 binding sites for low density lipoprotein; this number is in essential agreement with the number of sites computed on the bases of membrane-limited diffusion, 300.

The second part of this year's work involved a four-month detail with the Chemistry and Physics Division of the Food and Drug Administration, which has responsibility for estimating contamination of food by substances present in the packaging materials. I collaborated on the development of a classification system for these materials (which are mostly polymers) based on literature data on diffusion through polymers.

The remainder of the year's work involved initiation of a new study, concerning the rates of transmigration of phospholipids between the inner and outer halves of the phospholipid bilayer of cell membranes. This process does not occur at significant rates with either synthetic phospholipid bilayers or human erythrocytes, but does take place in rat erythrocytes. Since the overall structures of erythrocyte membranes in mammalian species are very similar, it is believed that the differences between human and rat erythrocytes reflect molecular level differences in membrane organization. To define these differences, transmigration rates will be studied with several other mammalian species, and also for erythrocytes whose membrane lipids have been artifically modified. Thus far, work on this problem has been limited to the extraction of a material necessary for these studies from beef liver, phospholipid exchange protein, and the development of experimental procedures for measuring the rates.

Publications:

Gottlieb, M. H.: Rates of cholesterol exchange between human erythrocytes and plasma lipoproteins. Biochim. Biophys. Acta, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-27006-23 LPB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Developmental biochemistry of insect metamorphosis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. Levenbook	Research Chemist	LPB-NIAMDD
OTHER:	A. Bauer	Research Physiologist	LPB-NIAMDD
	E. Zomer	Visiting Fellow	LPB-NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Comparative Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Physico-chemical characterization of the blowfly storage protein calliphorin has been undertaken. Extensive use of computer modeling (curve fitting) has allowed us to surmise which amino acid residues on the protein surface, and what type of forces, are involved in oligomer formation. These conclusions are in good agreement with those derived from chemical modification experiments. The first of three manuscripts relating to the above is completed, and the second started. Attempts to separate and to isolate calliphorin hexomers and/or monomers by polyacrylamide gel electrophoresis and/or isoelectric focusing have been abandoned.

PHS-6040
(Rev. 10-76)

Project No. Z01 AM-27006-23 LPB

Project Description:

Objectives:

(1) The role of tyrosine and lysine residues in the reversible association-dissociationof calliphorin oligomers.

(2) Identification of the types of pH sensitive forces involved in oligomer formation.

(3) Separation and isolation of individual calliphorin hexamers and monomers.

Methods Employed:

Iso-electric focusing, isotachophoresis, and various gel electrophoresis techniques; curve-fitting and computer modeling (MLAB).

Major Findings:

No real progress has been made in isolating individual, pure calliphorin hexamer or monomer species. Contrary to earlier reports, we now find as the result of improved separation techniques nine, and not six, individual monomeric species. This makes the isolation problem even harder. As a last resort in this direction, conditions have been worked out for separating the hexameric species by preparative isotachophoresis -- a new and novel procedure that we hope will lead to the desired isolation.

The reiterative, curve-fitting computer program (MLAB) has been used extensively for modeling calliphorin. MLAB coupled with infra-red, circular dichroism, and perturbation spectroscopy leads to the following conclusions:

(1) Hexameric calliphorin is composed of ~ 12% α-helix, ~ 14% β-pleated sheet and ~ 74% random structure. Conformational changes associated with dissociation to monomers is small; the amount of α-helix decreases only very slightly, and random structure increases to 80% of the total.

(2) Tyrosine residues are of major importance in the hexamer formation. In the associated hexamer, 7-8 tyrosines per monomer, with a normal pK of 10.0 are exposed, while upon dissociation an additional ~ 22 tyrosines with a pK of 10.9 now become exposed, and some 47 are buried. These data indicate that ~144 tyrosine residues participate in hexamer formation. Of the buried tyrosines, 5-6 with a $pK > 11.7$ are so deeply buried that they are not exposed even at pH 12 or at 65°C.

(3) Tryptophan residues do not appear to be involved in oligomer formation.

(4) Glutamic and aspartic acid carboxyl groups are fully ionized (COO^-) at all pH's above 6.5. They are thus unlikely to be responsible for the pH sensitive association-dissociation reaction.

Significance to NIAMDD Research:

Insect metamorphosis is a programmed process under hormonal control which entails the decay and destruction of larval cells concomitantly with the differentiation of cells and structures that will give rise to the adult insect. The greater part of this process takes place within the span of a week. This telescoping of the aging and development process in the insect might be expected to bring forth features otherwise unobservable in the corresponding processes of higher animals, which occur in a much more expanded time scale. In particular, the study of the fate of the principal soluble larval protein refers to the fate of an essential component of the developmental machinery including its conversion into new proteins during development and aging.

Proposed Course of Projects:

(1) Isolation of individual calliphorin hexamers and monomers for chemical characterization and a study of their interrelationships.

(2) The fate of injected labeled calliphorin during metamorphosis of the blowfly.

Publications:

Levenbook, L., Boctor, F., and Fales, H. M.: Biochemical studies of tick embryogenesis. Free sugars in adult hemolymph and during embryogenesis of Dermacentor andersoni. J. Insect Physiol. (In Press).

Levenbook, L., and Bauer, A. C.: Calliphorin and soluble protein of haemolymph and tissues during larval growth and adult development of Calliphora vicina. Insect Biochem. (In Press).

REPORTED IN PRESS - 1979

Pau, R., Levenbook, L., and Bauer, A. C.: Inhibitory effect of β-ecdysone on protein synthesis by blowfly fat body in vitro. Experientia 35: 1449-1451, 1979.

Levenbook, L.: Hemolymph volume during growth of Calliphora vicina larvae. Ann. Ent. Soc. Amer. 72: 454-455, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-27007-18 LPB

PERIOD COVERED October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Molecular Mechanism of Human Red Cell Sickling

Molecular Mechanism of Blood Coagulation (the initial stage of)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Murayama	Research Chemist	LPB-NIAMDD
OTHER:	A. Adams	Staff Fellow	LPB-NIAMDD

COOPERATING UNITS (if any) Prof. Ray Henry and Dr. Robert Nalbandian, Wayne State University, Detroit, Michigan; Prof. Ray Pepinsky, Univ. of Florida, Gainesville, Florida; Mr. Marvin Shapiro, DCRT-NIH, Bldg. 12, Bethesda, Md.

LAB/BRANCH Laboratory of Physical Biology

SECTION Comparative Physiology Section

INSTITUTE AND LOCATION NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-3/4	1-3/4	0

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Influence of hydrostatic pressure on the reversible polymerization of human sickle cell hemoglobin (which has a negative temperature coefficient of gelation) has been investigated in order to derive the volume of activation in the process. Parallel investigation has been done with blood clotting protein (fibrin), which has a positive temperature coefficient of polymerization. It has been found that the volume of activation for S hemoglobin polymerization is 100 cc per mole and for fibrin 70 cc per mole. The study must be extended further to include the following: effect of concentration of respective proteins, effect of pH, and temperature. This type of investigation will give us further insight into the nature of protein-protein interactions and the topological problems involved in the problem of polymerization still not well understood.

PHS-6040
(Rev. 10-76)

Project No. Z01 AM-27007-18 LPB

Project Description:

Objectives:

1. To investigate a molecular mechanism of the "unsickling" of erythrocytes from sickle cell anemia patients.

2. Ultrastructure of the sickled human erythrocyte.

3. Molecular volume change in the process of polymerization of fibrinogen and fibrin.

Methods Employed:

Aggregation-deaggregation of sickle cell hemoglobin by pressure, temperature and alkali halides and other hydrogen bond breaking or transferring agents: these are alcohols, amines, and urea derivatives.

Aggregation and deaggregation of fibrin polymer was investigated by high hydrostatic pressure, vacuum, and chemical agents which altered the rate of polymerization urea, piracetam, hexamethylenetetramine and those compounds which are similar to amino acid residues which might be involved in the process of polymerization.

Because high hydrostatic pressure slowed the rate of polymerization of deoxy-S hemoglobin, it occurred to us to ask what might happen to fibrin polymerization under the same condition. Our finding suggests that the volume of activation is a large positive number...70 cc $mole^{-1}$ when the ionic strength is 0.15 M but when the ionic strength is increased to 0.8 M NaCl then the ΔV^* is reduced in half -- to 35 cc $mole^{-1}$. In fact the pressure effect of fibrin polymer is very dramatic in that mirky gel melts almost instantaneously when the pressure is applied.

Magnetic contributions of three anisotropic elements: the heme groups, the aromatic groups and the alpha-helical segments were calculated from the 3-D coordinates of human hemoglobin molecule.

Major Findings:

1. Human fibrinogen precipitates at low temperature; in the process the volume of activation (ΔV^*) is 70 cc $mole^{-1}$.
2. Des-A fibrinogen aggregates with ΔV^* of 140 cc $mole^{-1}$.
3. Des-A&B-fibrinogen (fibrin) aggregates with ΔV^* of 700 cc $mole^{-1}$.
4. In the presence of 0.8 M NaCl instead of isotonic saline, the volume of activations are halved.
5. In vacuo (22 Torrs) the rate of fibrin aggregation is increased by 260% over the control; at 2,000 Psi, the rate of aggregation is reduced by -65% over the control.

9. With platelet-poor whole blood, ESR is very slow, either in vacuo or at one atmosphere.
10. When blood platelets are inactivated by 0.5 mM Piracetam ESR is slowed down by 100% over the control.
11. Deoxy-HbS aggregates with ΔV^* of $+91 \pm 15$ cc $mole^{-1}$.
12. Hydrostatic pressure inhibits gelation by increasing deoxy HbS solubility, which is $+8.6 \times 10^{-6}$ PSI^{-1}.

Significance to NIAMDD Research:

Sickle cell anemia is a hereditary, hemolytic anemia -- an inborn error of metabolism; hence exceedingly significant to NIAMDD mission. In addition to that problem of coagulation -- an investigation into fibrin polymerization at the molecular level is of utmost importance to all of us. I think that the understanding of the high pressure chemistry and physics to blood clotting is of importance particularly to those boys who must engage in deep-sea diving. It is known that the slightest injury is sufficient for some of the boys to bleed to death while in a decompression chamber; these fellows are literally in between the devil and the deep blue sea. Hexamethylenetetramine could be developed into an antidote for deep sea divers' bleeding problem.

Proposed Course of Project:

To follow through the effect of high hydrostatic pressure to prevent denaturation of human fibrinogen, yet still be able to kill hepatitis virus -- pressure-heat pasteurization of human blood products.

Now, the effect of high pressure on blood platelets are in order. The role played by blood platelets in hemostatsis is well known, but the effect of high pressure is still unknown; this will be investigated.

Publications:

1. Nalbandian, Robert M., Raymond L. Henry, and Makio Murayama: Sickle-cell Disease: two new therapeutic strategies, Lancet 570-571, 1978.

2. Murayama, Makio: Biochemical and biophysical effects of Piracetam on human erythrocytes. Proceedings of International Symposium on Neurotrophic Drug. October, 1979, Rio de Jeneiro, Brazil (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER 701 AM-27008-21 LPB

PERIOD COVERED: October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of metabolic activity in microorganisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. S. Kempner	Physicist	LPB	NIAMDD
OTHER:	J. H. Miller	Chemist	LPB	NIAMDD

COOPERATING UNITS (if any)

Dr. Frank Gaertner, Oak Ridge National Laboratories; Dr. Don Gruol, The Salk Institute; Dr. Thor Nielson, LNE-NIAMDD; Dr. Clifford Steer, DD-NIAMDD; Dr. Martin Rodbell, LNE-NIAMDD; Dr. Joan Harmon, DR-NIAMDD.

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SECTION
Comparative Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The relationships between biochemically sequential reactions in polyenzyme complexes is being examined. The use of radiation inactivation to analyze these systems and also the various membrane receptor systems is underway. Studies of the fundamental mechanisms of radiation damage to macromolecules are being conducted.

PHS-6040
(Rev. 10-76)

Project No. Z01 AM-27008-21 LPB

Project Description:

Objectives:

1. An understanding of the control of metabolic activity in organisms.

2. Analysis of the nature of macromolecular damage by ionizing radiation.

Methods Employed:

Determination of enzymatic activity in cell extracts after isolation of enzymatically active complexes. High energy radiation inactivation of proteins, membranes and other cell extracts.

Major Findings:

The effects of ionizing radiation on macromolecules has been studied for many years. It has been assumed that the fundamental physics of radiation action is the same as that previously determined in the early days of atomic physics. However, those important studies were all carried out with small molecules, principally diatomic gases and air. Analysis of the radiation effects on macromolecules have now revealed several distinctive phenomena. First, irradiation of macromolecules in liquid solution was found to be 1000 times more damaging than the same radiation dose delivered to lyophilized polymers. Second, in a large number of enzymes it was found that an ionization (caused by X-rays or fast electrons) anywhere in the polypeptide chain destroyed enzymatic activity, implying an energy transfer throughout the polymer chain, and third, energy transfer from one polypeptide chain to another was not observed. Finally, lyophilized or frozen proteins and nucleic acids at low temperature are relatively insensitive to ionizing radiation compared with the same materials irradiated at room temperature; similar observations have been made in effects of ionizing radiation on synthetic plastics. This temperature factor is quantitatively the same for all polymers even though these temperature effects included such diverse properties as photoconductivity of irradiated Teflon, hydrogen evolution of irradiated polyethylene, infectivity of irradiated viral DNA, and enzymatic activity of irradiated proteins. None of these four phenomena were observed or predicted from the original atomic physics studies.

The great radiation sensitivity of macromolecules in liquid solution has already been successfully explained: irradiation of the solvent (usually water) leads to the production of a variety of radicals and other reactive compounds which diffuse great distances and chemically react with the biologically active macromolecules. The other phenomena just described are as yet unexplained. There are several simple and plausible suggestions to account for these observations. It is assumed that the initial interaction between ionizing radiation and the target macro-

molecule is adequately explained by the classical picture: there is a collision between the radiation and an orbital electron. The invasive quanta or particle deposits (on the average) about 1500 kcal/mole at each encounter and then continues along its trajectory. Meanwhile, back at the molecule, the struck electron is ejected with some fraction of the energy (in the form of the kinetic energy of the electron). This leaves a positively charged atom and the remaining energy. This is capable of causing further ionizations, excitations and degradation ultimately as heat. Because of the close coupling of the molecular orbitals in a macromolecule, the energy can be transferred along the chain to other atoms before causing other ionizations. Similarly the ejected electron can interact with other atoms and spread energy by this mechanism. Because of the close packing of atoms in a polymer chain, the "secondary electrons" will see a large number of electron clouds and will interact -- sometimes ionizing -- atoms in the same chain. Only a few secondary electrons would be expected to escape from the original polymer and therebe free to interact with other chains. In view of the inverse square law, the likelihood of "hitting" an adjacent polymer chain is small. The explanation just given for transfer of energy along a single polymer chain was transmission by the secondary electrons and also by coupling of the molecular orbitals. The higher the temperature, the greater the overlap of electron orbitals of adjacent atoms in these polymers, and therefore the more efficient is the transfer of energy along the chain. At low temperature there is less efficient transfer and the "damage" does not spread as far down the chain. Loss of biological activity (i.e., infectivity of DNA, enzyme activity of a protein) can be due to direct damage on some "active site" or by some conformational change due to ionization elsewhere which results in slight shifting of atom positions in an active site leading to a great drop in catalytic efficiency. It then follows that the diminished transfer of ionization energy along the chain at low temperatures results in less observable biological damage compared to the effectiveness of the same dose of radiation at higher temperatures.

With this simple explanation, the three phenomena of radiation effects on polymers have been explained, perhaps successfully. Several experiments are in progress to test the validity of this description.

Publications:

Shehata, T. E. and Kempner, E. S.: Synchronization of division in vitamin B_{12}-starved Euglena gracilis. J. Protozool. 26: 626-630, 1979.

Kempner, E. S. and Dion, R. L.: Absolute DNA determinations by flow microfluorometry. J. Protozool. 26: 630-631, 1979.

Publications: (continued)

Harmon, J. T., Kahn, C. R., Kempner, E. S. and Schlegel, W.: Characterization of the insulin receptor in its membrane environment by radiation inactivation. J. Biol. Chem. 255: 3412-3419, 1980.

Kempner, E. S., Miller, J. H., Schlegel, W. and Hearon, J. Z.: The functional unit of polyenzymes: determination by radiation inactivation. J. Biol. Chem. In press.

REPORTED IN PRESS - 1979

Kempner, E. S.: Metabolic compartments and their interactions. In "Cell Compartmentation and Metabolic Channeling". Eds., L. Nover, F. Lynen and K. Mothes. VEB G. Fischer Verlag, Jena and North Holland Publ. Co., Amsterdam (joint publishers), 1980, pp. 217-224.

Schlegel, W., Kempner, E. S. and Rodbell, M.: Activation of adenylate cyclase in hepatic membranes involves interactions of the catalytic unit with multimeric complexes of regulatory proteins. J. Biol. Chem. 254: 5168-5176, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 27009-02 LPB

PERIOD COVERED October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Macromolecular Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alasdair C. Steven	Visiting Associate	LPB NIAMDD
OTHER:	Charles H. Hanna	Biol. Lab. Techn.	LPB NIAMDD

COOPERATING UNITS (if any)

Other collaborators on specific activities are identified below, in the accounts of these activities.

LAB/BRANCH Laboratory of Physical Biology

SECTION Section on Comparative Physiology

INSTITUTE AND LOCATION NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new facility is being established for structural studies on biological macromolecules and macromolecular assemblies by high resolution electron microscopy reinforced by optical diffraction and computer image processing. This facility is operational, due to a major laboratory renovation accomplished over the past year, and further development of the facilities is envisaged. Progress has been made on structural studies of the human erythrocyte membrane and a filamentous plant virus. The major results of the past year have come from a study of immunoglobulin crystals by a hybrid approach integrating electron microscopy and computer graphics with X-ray crystallography.

PHS-6040

Project Description:

Objectives: To elucidate the structural organization, design principles, and functional significance of macromolecular assemblies. Specific systems under study include biological membranes, virus particles and complexes crystalline in one, two, or three dimensions.

Experimental Approach: These objectives demand a unified approach, synthesizing information from diverse sources (biochemical, biophysical, ultrastructural, etc.) Our strategy is to establish the structural basis in projects which are usually collaborative in nature. At the level of structural techniques, we are engaged in

1) high resolution electron microscopy,
2) image analysis and enhancement by optical diffraction and computer image processing,
3) development of appropriate specimen preparation techniques,
4) thermodynamic studies (calorimetry).

Statement of Progress:

A: Laboratory Development

I. The major development of the past year was execution (December, 1979 - January, 1980) of the long-awaited laboratory renovation, which has allowed the commissioning of the following experimental resources:

(a) Installation of Philips EM400 electron microscope, with image intensifier, geometric stage, and radiation reduction kit. It is expected that the microscope will be fully operational by July, 1980.

(b) Relocation of Philips EM300 electron microscope, creating modular space for the optical diffractometer.

(c) Construction of optical diffractometer. This instrument was assembled according to a novel design, optimized for rapid visual scanning of large batches of electron micrographs, and for photographing high quality diffractograms. The instrument is already in operation but will be further refined over the next year, during which time a detailed account of the instrumental design will be prepared.

(d) Commissioning of new laboratory sub-module for preparative biochemical work.

(d) Computer software for image processing has been extended by developing programs to transfer digital images from the analysis programs implemented on the IBM 370 computer to the graphics hardware and analysis system (implemented by Dr. B. Trus, NIDR) on the PDP11/70 computer, and to the Optronics Film-Writer at Johns Hopkins Applied Physics Laboratory. The major advance represented by these developments is a substantial improvement in the quality and variety of photographic output of electron microscope images after digital image processing.

B. Research Projects (Summaries of Progress and Proposed Future Course)

I. Electron Microscopy of Immunoglobin Crystals (in collaboration with Dr. M. Navia, LMB, NIAMDD)

Using digital methods, we have filtered electron microscope images of negatively stained microcrystals of IgG-DOB, a human myeloma immunoglobulin. These images were then compared to "ideal" micrographs, derived computationally from the crystal structure which is known to a resolution of 0.6 nm from X-ray diffraction studies. The purpose of this study was to evaluate qualitatively and quantitatively the fidelity of structure representation in such electron microscope images. From a priori considerations, potentially severe perturbations of molecular structure might be caused by dehydration, by fixation, by electron irradiation, by chemical reaction with the stain, by multiple electron scattering, or by microscope aberrations. The central conclusion of this study is that, to an effective resolution of about 2.0 nm, the electron microscope images convey a remarkably accurate representation of the molecule. In future developments, it is intended to extend this approach with electron microscopic work on crystals which are too small, or otherwise unsuitable for analysis by conventional methods of X-ray crystallography.

II. Ultrastructure of Beetroot Yellow Necrotic Virus (BYNV)(in collaboration with Dr. M. Wurtz, University of Basel, Switzerland)

Plant viruses represent important, experimentally accessible, systems for studying the assembly of nucleo-protein particles. A combination of electron microscopy, optical diffraction and image processing has been used to determine the structure of BYNV. Our results on the structure of this filamentous virus particle show that the packing of protein subunits is based on a single-stranded right-handed helix which returns to its angular origin after four helical turns. The final ambiguity to be resolved in this study, now in its final stages, is to determine whether 53 or 57 protein subunits are included in these four helical turns.

III. Paracrystalline Tetragonal Particle Arrays in the Human Erythrocyte Membrane (in collaboration with Dr. M. Gottlieb, LPB, NIAMDD).

We have observed regular patches of tetragonally arranged particles (periodicity approximately 14 nm) in erythrocyte membranes incubated in distilled water. Our immediate goals are structural and chemical characterization of these particles, improvement of yield of these paracrystalline patches, and structural analysis of the several characteristically different appearances which these arrays project in negatively stained preparations. An interesting preliminary result of structural studies is that there appear to be several morphologically distinct classes of patch. We have not yet been able to identify the protein(s) which constitute these particles because of low yields, although SDS-polyacrylamide gel electrophoresis of the membranes on incubation show protein patterns characteristic of erythrocyte ghosts, modified by a moderate degree of limited proteolysis and partial release of the cytoskeletal proteins, spectrin and actin. To approach the identification question, we have raised antisera against spectrin, ankyrin, and band 3 proteins, as well as against sealed ghosts and inverted vesicles. In the next phase of this study these antisera will be used as immuno-electron microscopic probes. In the longer term, we intend to extend the structural analysis to higher resolution by developing techniques to produce larger, better-ordered arrays, and to explore possible functional implications of this aggregation phenomenon.

IV. Proteodynamics: Cooperative Conformational Changes in Protein Assemblies (in collaboration with Dr. L. W. Black, University of Maryland Medical School, Baltimore).

In earlier studies (1,2) we have characterized, from a structural viewpoint, major conformational changes which occur during the assembly of the proteins which constitute viral headshells. These changes are important to the assembly process, both in a physiological and in a regulatory sense. In order to analyze these events thermodynamically, we have started a series of calorimetric experiments (using a Perkin-Elmer DSC-2 micro-calorimeter kindly made available by Dr. Norman Gershfeld, LPB, NIAMDD) on protein prepared in the different conformational states. The parameters which we measure relate to thermal denaturation properties as a function of conformational state. In further studies we intend to make direct measurements of the heat exchanges associated with the conformational changes (equivalent to latent heat of the reaction).

1) A. C. Steven, R. Contene, U. Aebi, and M. K. Showe (1976). J. Mol. Biol. 106:187-221.
2) A. C. Steven, and J. L. Carrascosa (1978). J. Supramol. Struc. 10: 1-11.

Significance to Biomedical Research and the Program of the Institute:

An improved understanding of macromolecular assembly events--in particular, membrane biogenesis and virus propagation--should be of direct relevance to several areas of medical research.

Publications:

Rosenbusch, J. P., Steven, A. C., Alkan, M., and Ragenass, M.: Matrix Porin: A Periodically Arranged Pore Protein in the Outer Membrane of Escherichia coli. In Baumeister, W. (Ed.): Regular 2-D Arrays of Biomolecules: Structure Determination and Assembly. Berlin, Springer-Verlag, 1980, in press.

Steven, A. C. and Navia, M. A.: On the fidelity of structure representation in electron micrograph of negatively stained protein molecules. Proc. Nat. Acad. Sci. USA, in press.

Annual Report of the Laboratory of Molecular Biology

National Institute of Arthritis, Metabolism and Digestive Diseases

The Laboratory of Molecular Biology has as its principal goal the understanding of biological processes at the molecular level. The research areas under investigation include the structures and chemical properties of biologically important materials. These involve studies of the organization of DNA and proteins in chromatin, investigations of enzyme and immunoglobulin structures by x-ray diffraction, investigation of polynucleotide chemistry and structure by synthetic and spectroscopic methods, studies of polynucleotide structure by fiber diffraction methods, and theoretical studies on the mechanism of energy conversion in biology, muscle contraction, the statistical thermodynamics of polynucleotide - complementary monomer interactions, ion transport and biochemical kinetics. In addition, other investigations are concerned directly with biological processes. These include the mechanism of transformation by SV40, the investigation of hemoglobin S gelation, genetic recombination, DNA synthesis and protein synthesis. Important advances have been made in these areas during this year.

Chromatin Structure and Function

We have continued studies of chromatin structure. Having shown previously that histone-base interactions appear to be of little importance in stabilizing nucleosome structure, we have now examined the electrostatic interactions between histones and DNA phosphodiester groups. We find that in the 30% of the nucleosome DNA near the ends, fewer than 3 positive charges for each ten base pairs interact strongly with DNA. We have also begun studies of the higher order structure of chromatin. Using electric dichroism (in collaboration with E. Charney and D. Rau, Laboratory of Chemical Physics) we have shown that when nucleosomes are packed in the "30 nm fiber" characteristic of chromatin, their faces must be nearly parallel to the fiber axis. We have proposed a model for the 30 nm fiber. We have studied histone-nucleosome interactions as a model for nucleosome packing in the 30 nm fiber, and have obtained evidence that the histone amino-terminal tails are important in the interaction, as predicted by our model for the fiber structure. We have also studied the interactions of nucleosome core particles with the high mobility group proteins, HMG14 and 17. We have determined the stoichiometry and sites of binding, and shown that these proteins bind preferentially to nucleosomes containing the transcriptionally activated globin genes of chicken erythrocytes. In other studies, transcription of eukaryotic DNA and chromatin have been carried out using a newly developed *in vitro* system isolated from *Xenopus* oocyte germinal vesicles.

Three-Dimensional Structure of Proteins of the Immune System

X-ray diffraction data have been collected to 2.7A resolution by the oscillation camera method for the mouse immunoglobulin Fab McPC603. These data have been processed and are currently being used to provide a refined three-dimensional high resolution picture of the antibody combining site.

Similarly, data have been collected and processed for J539 a mouse galactan-binding Fab, to 2.5A resolution. These data are currently being used to extend the resolution of the crystal structure determination from 4.5A to 2.5A resolution.

Crystals obtained from a solution containing the pyruvylated-galactoside-binding human IgMFv, WEAVER, have been observed to undergo a novel transformation in the presence of $K_2Pt(CNS)_6$ to a higher symmetry space group. This result implies that the crystalline material is the variable domain from either the light or the heavy chain, but cannot consist of both together.

In collaboration with the Immunology Branch, NCI, procedures have been developed for isolating significant amounts of histocompatibility antigens from miniature swine. Milligram quantities of this material have been obtained and attempts are being made to crystallize it. Small crystals have been obtained and efforts are being made to grow them larger.

Three-Dimensional Structures of Enzymes

Crystals of the acid protease from Rhizopus chinensis have been used to collect further X-ray diffraction data to 2.0A resolution. The native enzyme and the pepstatin-inhibited enzyme have been refined to 2.5A resolution. An explanation for the molecular basis of pepstatin inhibition of acid proteases has been inferred.

Crystals of the enzyme S-adenosylmethionine synthetase from E. coli have been prepared that are of a form suitable for X-ray diffraction. Two distinct crystal forms have been observed depending on the conditions of crystallization

Energy Conversion in Biology, Especially Muscle Contraction

Four different topics have been studied under this project:

(a) Isometric transient and steady contraction properties have been calculated from a self-consistent theoretical model of muscle contraction. Agreement with experiment is very good. Recent biochemical results, however, compel us to extend the model to eight states (four attached, four unattached). We are presently engaged in adjustment of model parameters to obtain the best possible fit with experimental data. The unique feature of this work is the bridge we are building between muscle biochemistry and muscle biophysics.

(b) In preparation for future extension of the above model to the regulation of muscle contraction by Ca^{++}, we have developed a model for the cooperative steady-state ATPase activity of myosin subfragment-1 on actin-troponin-tropomyosin. Experiments (by Chalovich and Eisenberg) are in progress that will be compared with the theory.

(c) A much more detailed account has been published on the thermodynamic and kinetic interrelationships between the four main membrane systems of mitochondrial oxidative phosphorylation: respiratory chain; reverse ATPase; ADP-ATP exchange, and H^+, P_i symport. Also this treatment has been extended

in two ways: the tight coupling assumed between the two subforces and subfluxes for each of the four systems has been relaxed to allow for the experimentally indicated incomplete coupling (inefficiency); and the four systems in the model have been extended to eight, to allow for H^+ leak, Ca^{++} leak, Ca^{++}-H^+ exchange; and ATP consumption in various processes within the mitochondrial matrix.

(d) A comprehensive theoretical study has been made of the cooperative properties of a lattice of interacting three-state enzyme molecules. Also we have examined the limits of the validity of the assumption, in biochemical kinetics, that the discrete states of a biochemical cycle, operating at steady state, are themselves separately in an internal equilibrium among substrates.

Statistical thermodynamics of protein and polynucleotide systems

Four separate topics have been investigated here:

(a) The equilibrium binding constants of the myosin fragments S-1 and HMM onto an actin filament have been calculated, semiquantitatively, from statistical mechanical first principles. The relations between one-headed and two-headed binding are especially interesting. Agreement with experiment is quite satisfactory.

(b) The experimental data of Greene and Eisenberg on the cooperative equilibrium binding of S-1-ADP onto actin-troponin-tropomyosin, in the presence and absence of Ca^{++}, have been matched very well by a theoretical treatment that is a rather complicated extension of the well-known two-state, linear Ising model. The experiments and the theory are being extended to allow for different salt concentrations and for different nucleotides bound to the S-1.

(c) Steady-state head-to-tail polymerization of actin or of microtubules require a flux of ATP or of GTP, respectively. in this work, an explicit kinetic model was used to examine the bioenergetic aspects of this process. Also investigated, for an important special case, was the steady-state polymer length distribution. The distribution found is of the same form as found experimentally.

(d) In general, the structure of a covalently-closed circular DNA in solution depends on the bending and torsional stiffness of the double helix of the DNA molecule. The resistance of DNA to bending can be deduced from the experimentally determined DNA persistence length. In contrast, the torsional stiffness remains obscure. This is because the torisonal stiffness does not affect any properties of linear DNA molecules. It has been reported that theoretical studies of fluctuations of writhing numbers of model ring polymers could be used to estimate the torsional stiffness of DNA molecules. A necessary step in these studies is to generate a large number of ring polymers. In this current study, we have devised an efficient method of generating ring polymers (on lattice or off lattice) based on the "dimerization" method of Alexandrowicz. The torsional stiffness of DNA molecules will be studied using the ring polymers generated by this method.

Ion Transport Across the Nerve Axon Membrane

Recently, Fishman and his colleagues have found a low frequency feature in the admittance (or impedance) of squid axon membranes which cannot be accounted for by the usual Hodgkin-Huxley dormalism. Two physical processes which slightly modify the usual HH formalism have been proposed to explain this low frequency feature by Fishman and his colleagues. One process involves the addition of an inactivation to the HH K^+ kinetics. The other involves the K^+ accumulation outside the axon membrane. In our work, we have shown that the experimental low-frequency feature can be simulated easily with a "nonequilibrium" channel model. This model differs from the original Hodgkin-Huxley formalism and the modified models mentioned above in that this model requires an energy source to maintain a nonequilibrium steady state. In squid axons, the electrochemical gradients of K^+ and Na^+ may serve as the energy source.

Thermodynamics of Hemoglobin S Gelation

We have examined the thermodynamics of protein self-association, protein-ligand binding and protein-nucleic acid interactions and interpreted these with reference to the known thermochemistry of non-covalent interactions of small molecules.

We find that: 1) although there is variation in the magnitudes and signs of the enthalpy and entropy changes accompanying these associations, the widespread occurrence of negative enthalpy and entropy changes for self-association and ligand-binding of globular proteins can only be accounted for on the basis of hydrogen-bonding and non-covalent van der Waals' interactions taking place in an environment of low dielectric constant; 2) positive entropy changes arise from solvent molecules liberated upon association or from ionic and salt-bridge types of interactions; 3) the signs and magnitudes of the enthalpy and entropy changes results from the relative extent of the interactions described in (1) and (2). We have proposed a two-stage conceptual model for association processes based upon these two classes of interaction effects; 4) the characteristic temperature variation of thermodynamic parameters (increased enthalpic contribution at elevated temperature) is attributed to the diminution of the hydrophobic effect with increasing temperature.

This interpretation provides a qualitative understanding of the thermodynamics of protein association processes.

Structural Studies on Polynucleotides

X-Ray Crystallography

The aim of this project is to obtain structural information on polynucleotides and to provide insights into the factors which control structure, principally through X-ray fiber diffraction and model building studies. Current areas include 1) characterizing the secondary structures induced in

DNA by a variety of solvents and conditions, using new sample-handling techniques which give ordered diffraction patterns even in the presence of large amounts of solvent; 2) survey of RNA-DNA hybrid and RNA-RNA duplex structures in the presence of large amounts of solvent; 3) analysis of a unique structure adopted by an RNA-DNA hybrid, poly(rA)·poly(dT) in highly hydrated samples; 4) collaboration in a joint NMR and X-ray diffraction of natural and synthetic polynucleotides.

Poly 8-Methyladenylic Acid. A Novel Polynucleotide Forming a Single-Stranded Helix with Alternating Syn and Anti Conformations.

We had previously synthesized the first syn polynucleotide, poly (8BrA), in which all residues were constrained to the syn conformation by steric hindrance of the Br atom. The polymer forms a double-stranded helix of high stability. We have now found that changing the 8-subtituent from Br to CH_3, reducing the size slightly and the polarizability significantly, has a profound effect on properties of the polymer. Poly(8MeA) forms very cooperatively a single-stranded helix of moderate stability in which the bases are evidently arranged in a regularly alternating pattern of syn and anti conformations.

This is the first report of a regular single-stranded helix in aqueous solution and of a homopolymer with alternating syn and anti conformations. It resembles one aspect of the Z form of DNA, however, in which the copolymer (dG-dC)n has all purine residues in syn and pyrimidines in anti conformation. The CD spectrum of poly(8MeA) suggests that the structure is right handed.

Poly(I) Helices. Specific Structural Role of Alkali Metal Ions.

We had discovered previously that alkali metal ions form size-specific complexes with poly(I) by coordinating to specific sites in the axial channel of the 4-stranded helix. Further work has shown that both rate and extent of reaction are functions of specific ion concentration (this effect can be distinguished from an ionic strength effect by use of non-specific ions to provide counterion screening) as well as polymer concentration. These observations can evidently account for conflicting results obtained by different investigators of helical poly(I).

Effect of $NaClO_4$ on Stoichiometry of A-U Interactions.

A recent paper by Gukovskaya, Gukovsky, Chikh, and Sukhorakov (Biopolymers 19, 454 (1980) has reported that in $NaClO_4$ solutions the combining ratio of adenosine and poly (U) is changed from 1:2 (as we and others have observed in NaCl solution) to 1:1, and that 1:2 mixtures of poly (A) and poly (U) consist of both double and triple helices in $NaClO_4$. This paper has surprised most workers in the field, with opinion apparently divided as to whether it could be correct. We have reinvestigated these systems and find conclusive evidence that the combining ratio is not changed by perchlorate ion and is 1:2 in both cases.

In pursuing this work we have developed a method for obtaining binding isotherms from wash-out coutinuous ultrafiltration experiments. The method is much more rapid and convenient that equilibrium dialysis but produces the same results in the adenosine-poly(U) system studied above.

Protein Synthesis in Eukaryotes: Mechanisms and Metabolic Controls

The induction by gibberellic acid (GA) of α-amylase in the aleurone cells of barley is being studied as a model system for hormonal control of specific gene expression. We have shown previously that the addition of GA to these nondividing cells causes an increase in the amount of translatable mRNA for α-amylase. If cycloheximide was added along with GA, the yield of α-amylase mRNA from aleurones was greatly diminished. If cycloheximide was added at 2, 4, or 6 hours after GA, there was a gradual increase in the recovery of α-amylase mRNA. It appears that some protein(s) synthesized early after the addition of GA is needed for the appearance of functional α-amylase mRNA. Immunoprecipitation of the translation products of mRNA from cells treated with GA only, or with GA and cycloheximide, revealed that in the latter case a protein related to α-amylase, but migrating slower on SDS gels, was formed. Since both preparations of mRNA were translated in identical cell-free systems, the two immunoprecipitable proteins are most likely the result of different but related mRNA. These data suggest that the protein(s) whose synthesis is required for maximal expression of the α-amylase gene might be involved in the processing of precursor RNA. cDNA prepared from partially purified α-amylase mRNA is being used to clone α-amylase gene sequences. These DNA probes will facilitate the search for putative precursors of α-amylase mRNA.

Genetics and Structure of the Oncogenic Virus, SV40

The 90K protein of SV40 appears to be required for the maintenance of transformation since many cell lines transformed by viral strains containing a temperature sensitive mutation in this protein (tsA mutants) are temperature sensitive for their transformed phenotype. On the other hand many cell lines are not temperature sensitive. We have examined the integration patterns of SV40 into the host in the temperature-sensitive and insensitive lines. Although some correlation between copy number and temperature sensitivity exists, there is not a simple correlation.

In separate experiments we have shown in permissively infected monkey cells that SV40 replicates > 98% from its normal origin, ∿ 1% from a second origin 180^{o} around the genome and possibly as a rare event from other sites. When tsA mutants are shifted to the nonpermissive temperature, replication from the normal sites is eliminated and initiation appears to start randomly on the SV40 genome.

Replication of Colicin E1 Plasmid DNA

Closed circular DNA of the plasmid ColE1 of Escherichia coli can be replaced in vitro by a soluble enzyme system. Replication starts most frequently at any of the three consecutive nucleotides (dA, dA and dC) located at a unique position on the DNA. No plasmid encoded protein is

required for this replication. Initiation of replication can be demonstrated with a mixture of three highly purified E. coli enzymes: RNA polymerase, DNA polymerase I and ribonuclease H (RNase H), an enzyme that creates a 3'-OH end by endonucleolytic cleavage of RNA which is hybridized to DNA. Previously we proposed that RNA polymerase synthesizes a transcript which is processed by RNase H and then used as a primer for DNA synthesis by DNA polymerase I. To prove the hypothesis, we have constructed a plasmid (pNT7) that consists of an 812 base pair segment containing the replication origin of plasmid ColE1 and of a 1240 base pair segment containing a β-lactamase gene. Transcription of the plasmid DNA showed that the plasmid DNA has three principal sites where transcription initiates in vitro. One is located in the ColE1 segment 555 nucleotides upstream from the origin. Most transcription from this site extends past the origin; the 3' portion of some of these transcripts hybridizes spontaneously to the template DNA. When RNase H is added in the reaction, the hybridized transcripts are cleaved by the enzymes in the origin region. When DNA polymerase I is included in the reaction along with RNA polymerase and RNase H, dAMP or dCMP is added directly onto the cleaved RNA molecules, many of which have an intact 5' terminus. The addition of a deoxyribonucleotide to the cleaved RNA can be regarded as the first step of ColE1 DNA synthesis. Once it has served as a primer, the RNA is eliminated from the product by RNase H. These results support our hypothesis and reveal, for the first time, a mechanism of RNA priming in initiation of replication of double-stranded DNA.

Functions Involved in Genetic Recombination

DNA Gyrase. Recent work on DNA gyrase has focused on the mechanism of the DNA supercoiling reaction. We have shown that the enzyme works by passing a DNA segment through a transient double-strand break in DNA in an asymmetric way. This mechanism is deduced from two kinds of experimental results: 1) DNA gyrase can unlink catenanes of double-stranded DNA and can also untie knotted DNA; 2) the DNA gyrase-induced supercoiling reaction changes the linking number of DNA in steps of two.

We have also isolated a fragment of the gyrase B protein that apparently coexists in E. coli with the whole protein. This fragment is about half the size of the gyrase B protein, and can complement the gyrase A protein to produce a DNA-relaxing (but not supercoiling) activity.

The role of DNA gyrase in regulatory processes was also investigated. In collaboration with G. Zubay's group at Columbia University we have shown that, in a cell-free E. coli system, transcription from different promoter sites has very different sensitivities (over a tenfold range) to inhibition of DNA gyrase. We speculate that changes in the intracellular level of DNA supercoiling could alter the transcription of widely scattered operons in parallel.

Integrative Recombination of Phage λ

We have previously shown that a sequence more than 200 base pairs long is involved in the specific recognition of the phage attachment site of bacteriophage λ. We have now generated shortened bacterial att DNA and cloned these

DNA fragments. By characterizing such DNA we found that a DNA molecule carrying the 15 base pair long common core sequence retained the capacity to act as the bacterial attachment site (att B). However these DNA molecules were less active than molecules that carried two additional 4 base pair sequences of the original DNA on both sides of the common core.

The nature of the cross-over point within the common core sequence during the integrative recombination reaction has also been investigated. A new method was developed to approach this problem, using the disintegration of ^{32}P atoms incorporated in a DNA molecule to generate base specific cleavage within a segment of DNA that derives from one of the parents of the reaction. Integrative recombination was shown to involve staggered cuts at unique sites within the common core sequence.

Novel Recombination Systems

Regulation of IS1-mediated transposition in E. coli is being studied. Of 50 compounds tested, acetate appears to enhance transposition of Tn9 (IS1-cat-IS1). UV irradiation of λ::Tn9 reduces transposability exponentially; however the reduction of IS1-cat-IS1 transposition is less than of IS1-λ-IS1 transposition. The expectation that portions of the E. coli chromosome should behave as transposons flanked by IS1 has not been borne out by experiment. One possibility is that resident chromosomal copies of IS1 are inactive.

Role of specialized recombination and IS elements in E. coli

In last year's report, we described mutants which are impaired in the F-promoted conjugal mobilization of pBR322, a process which is dependent upon the transposition of the IS element γδ (Tn 1000). Further work on these mutants, done in collaboration with the laboratory of K. Brooks Low at Yale University, has led to the hypothesis that the lesion is in an E. coli function required for the resolution of plasmid cointegrate structures which have been proposed to be intermediates in the transfer of pBR322 and, more generally, in the transposition of IS elements.

We have also studied the effect of recA-dependent recombination between γδ element on the plasmid F and the E. coli chromosome. These studies, done in collaboration with the laboratory of Joan Steitz at Yale University, have demonstrated that γδ sequences in the chromosome of E. coli K12 are the classical sex factor affinity (sfa) sites of F-chromosomal interaction.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33000-14 LMB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Study of Functions Involved in Genetic Recombination

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Martin Gellert,	Chief, Met. Enzymes Sec.,	LMB NIAMDD
Other:	Kiyoshi Mizuuchi	Visiting Scientist	LMB NIAMDD
	L. Mark Fisher	Guest Worker	LMB NIAMDD
	Mary O'Dea	Chemist	LMB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Metabolic Enzymes

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3 1/3	2 1/3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

DNA Gyrase. Recent work on DNA gyrase has focused on the mechanism of the DNA supercoiling reaction. We have shown that the enzyme works by passing a DNA segment through a transient double-strand break in DNA in an asymmetric way. This mechanism is deduced from two kinds of experimental results: 1) DNA gyrase can unlink catenanes of double-stranded DNA and can also untie knotted DNA; 2) the DNA gyrase-induced supercoiling reaction changes the linking number of DNA in steps of two.

We have also isolated a fragment of the gyrase B protein that apparently coexists in E. coli with the whole protein. This fragment is about half the size of the gyrase B protein, and can complement the gyrase A protein to produce a DNA-relaxing (but not supercoiling) activity.

Project Description:

DNA Gyrase In the past year, we have made considerable advances in understanding the mechanism of the DNA supercoiling reaction induced by DNA gyrase. Recent work from our laboratory and others has shown that the enzyme passes a DNA segment through a transient double-strand break at another place in the DNA and then reseals the break. In effect, there is active transport of a DNA double helix through itself.

Evidence for this reaction has come from two types of experiments. First, we have shown that DNA gyrase can unlink catenated (interlocked) circles of double-stranded DNA and can also unknot knotted DNA molecules. Both these DNA species are made with the phage λ integrative recombination reaction, using DNA substrates that contain the phage and bacterial attachment sites in a single supercoiled DNA molecule. If the attachment sites are in the same orientation, recombination makes catenanes; if the sites are in opposite orientation, knots are made. Incubation of these species with DNA gyrase and ATP leads to separation of catenanes or to unknotting, as can be seen by either gel electrophoresis or electron microscopy.

To convert a catenane to two separate circles requires a transient double-strand break in the DNA; so does the resolution of a knotted molecule to a simple circle. This shows that DNA gyrase can make transient double-strand breaks. To show that it does so as part of the normal supercoiling reaction, we used a different experiment. Passage of a DNA segment through a double-strand break within a circular DNA will change the number of superhelical turns in the DNA (technically termed the linking number) in steps of two, rather than steps of one as would be caused by rotation around a single-strand break. By use of DNA samples with a unique linking number, we have shown that DNA gyrase action does indeed change the linking number in steps of two. Based on these observations, a mechanistic model for the reaction was proposed, with asymmetric wrapping of DNA on the enzyme being followed by transfer of the wrapped DNA segment through the enzyme-DNA complex. This second step is coupled to the binding of ATP; hydrolysis of ATP then releases the translocated DNA segment in preparation for another cycle of reaction.

In a separate study, a protein has been isolated from E. coli that appears to be a fragment of the DNA gyrase B protein. This protein can complement the gyrase A protein to produce a DNA-relaxing activity, but cannot cause DNA supercoiling. Apparently a part of the gyrase B protein needed for energy coupling has been cleaved off.

Publications: Gellert, M., Mizuuchi, K., O'Dea, M.H., Ohmori, H., and Tomizawa, J.: DNA gyrase and DNA supercoiling. Cold Spr. Harb. Symp. Quant. Biol. 48: 35-40, 1979.

Gellert, M., Fisher, L.M. and O'Dea, M.H.: DNA gyrase: Purification and catalytic properties of a fragment of gyrase B protein. Proc. Natl. Acad. Sci. USA 76: 6289-6293, 1979.

Mizuuchi, K., Fisher, L.M., O'Dea, M.H. and Gellert, M.: DNA gyrase action involves the introduction of transient double-strand breaks into DNA. Proc. Natl. Acad. Sci. USA 77: 1847-1851, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33001-11 LMB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Protein Synthesis in Eukaryotes: Mechanisms and Metabolic Controls

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Co-P.Is.: Elizabeth S. Maxwell	Research Chemist	LMB NIAMDD
Subbaratnam Muthukrishnan	Visiting Scientist	LMB NIAMDD

COOPERATING UNITS (if any)

Seed Laboratory, USDA, Beltsville, Md. (Dr. G. R. Chandra)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Metabolic Enzymes

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range objective of this project is to understand at the molecular level how proteins are synthesized in eukaryotes and how the process of protein synthesis is controlled at the levels of translation and transcription. The topics of current interest are: 1) the isolation and characterization of biologically active messenger RNA from eukaryotic cells. 2) The translation of eukaryotic mRNA in cell-free systems. 3) The identification and purification of immunoprecipitable forms of specific proteins synthesized in cell-free systems. 4) The induction of mRNA for specific proteins by plant hormones. 5) The partial purification of specific induced mRNA from barley. 6) The cloning of the gene sequences for α-amylase, and eventually for other hormone-induced enzymes, in the barley genome. 7) The use of DNA probes to study the expressi of specific genes in barley. 8) The possible processing of pre-mRNA for α-amylase.

PHS-6040
(Rev. 10-76)

Project Description: Z01 AM 33001-11-LMB

We have investigated gibberellic acid-induced changes in RNA isolated from aleurone layers of deembryonated barley seeds. This system has several features which make it attractive for the study of hormone action and gene activity. The aleurone cells are the only viable cells in deembryonated seeds. They are nondividing, and they synthesize large amounts of a limited number of proteins in response to the exogenous addition of the plant hormone gibberellic acid. We examined the effects of gibberellic acid by following the quantitative changes in total RNA, functional mRNA, and the capacity of these RNAs to direct the synthesis of proteins in general, and α-amylase specifically, under standard in vitro conditions.

Our results show that there is a continuing increase in total RNA as well as functional mRNA in aleurone cells during the first 12 hrs after gibberellic acid addition. This enhancement of functional mRNA is not the result of activation of an inactive pool of uncapped mRNA in the mature seeds. Inhibitors of RNA synthesis, such as cordycepin, reduce the amount of translatable mRNA that can be extracted, also suggesting that gibberellic acid-induced protein synthesis requires the continuing synthesis of mRNA.

Somewhat surprisingly, the induction of the synthesis of α-amylase mRNA depends on protein synthesis, since it is blocked by cycloheximide. If cycloheximide was added along with GA, the yield of α-amylase mRNA from aleurones was greatly diminished. If cycloheximide was added at 2, 4, or 6 hours after GA, there was a gradual increase in the recovery of α-amylase mRNA. It appears that some protein(s) synthesized early after the addition of GA is needed for the appearance of functional α-amylase mRNA. Immunoprecipitation of the translation products of mRNA from cells treated with GA only, or with GA and cycloheximide, revealed that in the latter case a protein related to α-amylase, but migrating slower on SDS gels, was formed. Since both preparations of mRNA were translated in identical cell-free systems, the two immunoprecipitable proteins are most likely the result of different but related mRNA. These data suggest that the protein(s) whose synthesis is required for maximal expression of the α-amylase gene might be involved in the processing of precursor RNA. cDNA prepared from partially purified α-amylase mRNA is being used to clone α-amylase gene sequences, These DNA probes will be used to search for putative precursors of α-amylase mRNA, to study the expression of the α-amylase gene, and to determine the number of α-amylase genes in the barley genome.

Publications: Chandra, G. R., Muthukrishnan, S. and Maxwell, E.S.: Hormonal regulation of genome activity in higher plants. In Mandava, N. Brushan (Ed): Plant Growth Substances. ACS Symposium Series 111, 1979, pp. 245-261.

Muthukrishnan, S., Chandra, G.R. and Maxwell, E.S.: Hormone-induced increases of functional mRNA and α-amylase mRNA in barley aleurones. Proc. Natl. Acad. Sci. USA 76: 6181-6185, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33005-04 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Structural features of mRNA and their influence on translation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Subbaratnam Muthukrishnan	Visiting Scientist	LMB NIAMDD
Other:	Elizabeth S. Maxwell	Research Biochemist	LMB NIAMDD
	Marjan Pleij	Guest Worker	LMB NIAMDD

COOPERATING UNITS (if any)
Institute of Biochemistry & Biophysics, Warsaw, Poland (Dr. W. Filipowicz)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Metabolic Enzymes

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33006-02 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

In vitro study of the site specific recombination of phage lambda.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: K. Mizuuchi Visiting Associate LMB NIAMDD

Other: M. Mizuuchi Guest Worker LMB NIAMDD

COOPERATING UNITS (if any)

None

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Laboratory of Molecular Biology

SECTION
Section on Metabolic Enzymes

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-2/3	2/3	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have previously shown that a sequence more than 200 base pairs long is involved in the specific recognition of the phage attachment site of bacteriophage λ. We have now generated shortened bacterial att DNA and cloned these DNA fragments. By characterizing such DNA we found that a DNA molecule carrying the 15 base pair long common core sequence retained the capacity to act as the bacterial attachment site (att B). However these DNA molecules were less active than molecules that carried two additional 4 base pair sequences of the original DNA on both sides of the common core.

The nature of the cross-over point within the common core sequence during the integrative recombination reaction has also been investigated. A new method was developed to approach this problem, using the disintegration of ^{32}P atoms incorporated in a DNA molecule to generate base specific cleavage within a segment of DNA that derives from one of the parents of the reaction. Integrative recombination was shown to involve staggered cuts at unique sites within the common core sequence.

Project Description:

Integrative recombination reaction of phage λ is a reaction in which two specific DNA sites (att P and att B) are recombined to produce two hybrid sites (att L and att R). This reaction leads to the integration of the phage genome into its host E. coli chromosome in the lysogenic pass way of the phage life cycle. We have been studying this reaction by using an in vitro reaction system.

Previously we have shown that att P site must be on a supercoiled DNA molecule to be an efficient substrate. Molecular form of att B site is not critical. There is a 15 base pair common core sequence shared among the att sites in which the crossover reaction takes place. A stretch of DNA sequence extending about 230 base pairs that include the core sequence is involved in the recognition of phage site (att P).

On the other hand, more recently, we found that a stretch of 23 base pair sequence centered by the 15 base pair core contains essential information for the function of att B site. Furthermore, we found that a DNA molecule that contains only the core sequence derived from original att B retains a partial activity to act as att B.

We found the reaction involves a very conservative mechanism. No degradation or re-synthesis of the parental DNA is involved in the reaction. When a covalently closed circular form of the substrates are used, a large portion of superhelicity present in the substrate is preserved through the reaction process. No high energy co-factor is required for the reaction.

The nature of the crossover point within the common core sequence in the recombination reaction was investigated. To approach this question we developed a new method. Disintegration of ^{32}P atom incorporated in DNA was used to generate site specific cleavage of DNA molecule. A fragment of DNA carrying att B was labeled with ^{32}P and recombined with unlabeled att P DNA. Restriction fragments carrying recombination products (att L and att R) were purified and stored to arrow ^{32}P atoms to disintegrate. Resulting cleavage products were analyzed by gel electrophoresis. From the distribution of the size of cleaved products, the crossover point of each strand was determined. The reaction was shown to involve a cut at a unique point on each strand which is staggered from each other.

Studies on more detailed mechanism of the reaction is in progress.

Publications:

Mizuuchi, K. and Mizuuchi, M.: Integrative Recombination of Bacteriophage λ: in vitro study of the intermolecular reaction. Cold Spring Harbor Symp. Quant. Biol. 48: 1111-1114, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33051-08 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mammalian Enzymes Involved in Repair or Replication of DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	S. B. Zimmerman	Research Chemist	LMB NIAMDD
Other:	B. H. Pheiffer	Chemist	LMB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.2	0.6

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to identify and characterize enzymes involved in the repair and replication of DNA in higher organisms. Currently we are 1) Examining the ability of a eukaryotic DNA ligase to seal single strand breaks in chromatin; 2) continuing to survey eukaryotic sources for a DNA gyrase-like activity.

PHS-6040

Project Description:

Objectives: To isolate and characterize enzymes from mammalian sources which act on DNA, such as DNA kinase and DNA ligase, in order to attempt to understand more fully the processes of DNA repair and replication.

Methods: Standard techniques of biochemistry.

Major Findings: We wish to determine whether damaged sites in DNA tend to be localized with respect to nucleosomes in reconstituted chromatin, and also the influence such localization has one the enzymatic repair of these sites. The first DNA lesions being tested are single-strand breaks (nicks). The DNA is ^{32}P-labelled at chain termini; about 70% of this ^{32}P is at nicks and the remainder at ends and gaps. A number of attempts have failed to remove the 30% of the ^{32}P at non-nick termini. This non-ligateable fraction prevents unambiguous localization of nicks relative to nucleosomes, so that the question of phasing by nicks cannot be satisfactorily answered. We propose to use an alternative DNA substrate to solve this problem (see below).

Ligation of chromatin containing ^{32}P-labeled nicks clearly yields the ligase product within nucleosome-like structures as judged by DNase I digestion products and nucleosome isolation on sucrose gradients. Reconstruction experiments are consistent with the sealing reaction taking place while the nicks are in the nucleosome, rather than on free DNA followed by association with histones to yield a nucleosomal location of the ligase product. Again, it should be possible to establish this point more definitively using the specifically nicked DNA substrate described below.

We have made further attempts, uniformly unsuccessful, to demonstrate a DNA gyrase activity in eukaryotic cell preparations.

Significance to Bio-medical Research and the Program of the Institute: The enzymes discussed above are thought to function in repair and replication of DNA. Damaged regions in DNA have been implicated in a variety of disease states as well as in ageing and differentiation. Enumeration and characterization of the enzymes which carry out repair and replication will hopefully lend insight into the mechanisms of these processes and the aberrations in them which occur in various diseased states. Extension of these studies to repair in chromatin should more closely approach the *in vivo* state of DNA in higher organisms.

Proposed course; We will prepare unique sequence DNA (ϕX174) containing a single ^{32}P-labeled nick at a specific locus. Reconstitution of this DNA with the core histones followed by DNase I digestion and examination of the products on a single-strand gel should give an unambiguous assessment of phasing by nicks. If phasing does occur, as our earlier experiments have suggested, it should be possible to do the ligation experiment in a way which eliminates the uncertainly of whether the enzyme only acts on free stretches of DNA generated by sliding or dissociation of nucleosomes. This latter experiment is possible because we can recut specifically at the ligated site using the restriction enzyme originally used in the preparation of the

labeled DNA. The DNase I pattern with such material will only give bands if the sealed nicks are at specific locations relative to the nucleosomes, i.e. if no sliding or dissociation has occurred.

Publications: Pheiffer, B. H. and Zimmerman, S. B.: DNA kinase from nuclei of rat liver: mechanism reversal and inhibitors of the reaction. Biochemistry 18: 2960-2963, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33053-08 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Thermodynamics of Hemoglobin S Gelation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	P. D. Ross	Research Chemist	LMB NIAMDD
Other:	S. Subramanian	Visiting Associate	LNE NIAMDD

COOPERATING UNITS (if any)

None

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Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have examined the thermodynamics of protein self-association, protein-ligand binding and protein-nucleic acid interactions and interpreted these with reference to the known thermochemistry of non-covalent interactions of small molecules. We find that: 1) although there is variation in the magnitudes and signs of the enthalpy and entropy changes accompanying these associations, the widespread occurrence of negative enthalpy and entropy changes for self-association and ligand-binding of globular proteins can only be accounted for on the basis of hydrogen-bonding and non-covalent van der Waals' interactions taking place in an environment of low dielectric constant; 2) positive entropy changes arise from solvent molecules liberated upon association or from ionic and salt-bridge types of interactions; 3) the signs and magnitudes of the enthalpy and entropy changes results from the relative extent of the interactions described in (1) and (2). We have proposed a two-stage conceptual model for association processes based upon these two classes of interaction effects that provides a qualitative understanding of the thermodynamics of protein association processes.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To gain a basic physical-chemical understanding of the sickling phenomenon and to interpret the behavior of potentially therapeutic additives in terms of their effects upon the kinetics and thermodynamics of gelation. Specifically: 1) quantitative evaluation of the extent of non-ideality of hemoglobin both in solution alone and in multicomponent systems. 2) A systematic survey of the effect of anions, cations and organic solutes upon the solubility of deoxyhemoglobin S so that an idea may be gained of the relative importance of the ionic, hydrophobic and hydrogen-bonding interactions stabilizing the microtubular structure of deoxyhemoglobin S and requirements for more effective inhibitors of gelation be determined. 3) *In vitro* and *in vivo* evaluation of potentially therapeutic solubilizers of HbS.

Significance: The research summarized above has led to an increased understanding of the forces involved in protein self-association processes and protein-ligand binding. Since thermodynamic effects determine the solubility and dominate the kinetics of gelation of sickle cell hemoglobin, we believe this new knowledge can indicate which types of interactions to emphasize in the design of more effective inhibitors of hemoglobin polymerization.

Proposed Course of Project: Solubility studies aimed at finding more effective inhibitors will be continued. Attempts to verify our previous mechanism of non-covalent inhibition of gelation will be pursued by spectroscopic and X-ray crystallographic methods.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33054-05 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structural Studies on Polynucleotides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	S. B. Zimmerman	Research Chemist	LMB NIAMDD
Other:	B. H. Pheiffer	Chemist	LMB NIAMDD

COOPERATING UNITS (if any)
H. Shindo, CP, NIAMDD and J. Wooten, IRP, NICHHD

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.8	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to obtain structural information on polynucleotides and to provide insights into the factors which control structure, principally through X-ray fiber diffraction and model building studies. Current areas include 1) characterizing the secondary structures induced in DNA by a variety of solvents and conditions, using new sample-handling techniques which give ordered diffraction patterns even in the presence of large amounts of solvent; 2) survey of RNA-DNA hybrid and RNA-RNA duplex structures in the presence of large amounts of solvent; 3) analysis of a unique structure adopted by an RNA-DNA hybrid, poly(rA)·poly(dT) in highly hydrated samples; 4) collaboration in a joint NMR and X-ray diffraction study of natural and synthetic polynucleotides.

PHS-6040

Project Description:

Objectives: Obtain structural information on polynucleotides and provide insights into the factors which control structure.

Methods: Standard techniques of X-ray diffraction and biochemistry; new methods of sample preparation to examine oriented samples in the presence of excess solvent by X-ray diffraction; computerized molecular model building.

Major Findings: It has previously been proposed that several organic solvents and concentrated salt solutions convert DNA into the C form. This assignment is based upon the similarity of the circular dichroism spectrum of DNA in these media to the spectrum attributed to the C form. We have looked for the presence of the C form in DNA fibers immersed in these media, using the characteristic X-ray diffraction pattern as an assay for C form DNA. In most cases, however, the DNA adopted the B form rather than the C form. This discrepancy appears to arise from an incorrect assignment of the circular dichroism spectrum for the C form. DNA fibers immersed in certain media clearly do adopt the C form based on their diffraction patterns. In several cases, these C form structures have a greater helical rotation per residue than those which had been noted previously by Marvin et al. [J. Mol. Biol. 3, 547-565 (1961)], and so extend the range of structures which may be termed C form. The media surveyed included concentrated solutions of salts (NaCl, LiCl, NH_4Cl, KCl or guanidinium·HCl) as well as a number of organic solvents (methanol, ethanol, t-butanol, ethylene glycol or glycerol) either alone or in mixtures with water. The behavior of the sodium and lithium salts of DNA were compared in several instances.

Diffraction patterns from fibers of a number of RNA-RNA and RNA-DNA duplexes have been collected in the presence of an excess of solvent. In contrast to earlier reports in the literature made on samples under less solvated conditions, at least one clear example has been found of an RNA-DNA hybrid, poly(rA)·poly(dT), which adopts a B DNA-like conformation. A stereochemically acceptable model has been built which fits the observed diffraction pattern. The secondary structure of RNA-DNA hybrids is potentially of important functional significance in such processes as transcription and replication, hence it will be of considerable interest to determine the generality of the new structure for poly(rA)·poly(dT).

A joint NMR and X-ray diffraction study has been carried out with Drs. Shindo and Wooten. X-ray diffraction measurements were used to assess molecular conformation and orientation to aid in the interpretation of solid state NMR measurements on DNA fibers in the A, B and C forms. The NMR data indicate significant backbone variations are occurring, and that molecules in the B form undergo considerable rotational motion about their axes. Measurements on poly(dAdT)·poly(dAdT) also indicate backbone variation.

Significance to Bio-Medical Research and the Program of the Institute: Polynucleotides are crucial in controlling cells in both normal and diseased states. Understanding of polynucleotide structure will hopefully be of aid in understanding and interacting with cells in such diseased states. The

new sample-handling techniques are a step in this direction, allowing structure evaluation under conditions which are more like those which occur in vivo.

Proposed Course: There are many interactions which DNA and other polynucleotides undergo which can be profitably examined by the new sample-handling techniques which were developed above. For example, the survey of RNA-RNA and RNA-DNA duplexes under highly solvated conditions will be continued to determine the generality of a B DNA-like conformation for RNA-containing duplexes. The solvent-induced transformations of poly(dGdC)·poly(dGdC) will be tested; this unique polymer is apparently a left-handed duplex under certain conditions, so that its ability to adopt the B form while in fibers may help settle the hand of the DNA helix in the B or solution form as well as in the A form.

Publications:

Zimmerman, S. B. and Pheiffer, B.H.: A direct demonstration that the ethanol-induced transition of DNA is between the A and B forms: An X-ray diffraction study. J. Mol. Biol. 135: 1023-1027, 1979.

Davies, D.R. and Zimmerman, S.B.: A new twist for DNA? Nature 283: 11-12, 1980.

Shindo, H., Wooten, J.B., Pheiffer, B.H. and Zimmerman, S.B.: Nonuniform backbone conformation of DNA indicated by ^{31}P NMR chemical shift anisotropy. Biochem. 19: 518-526, 1980.

Shindo, H. and Zimmerman, S.B.: Sequence-dependent variations in the backbone geometry of a synthetic DNA fiber. Nature 283: 690-691, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33055-05 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Thermodynamics of Dehydrogenase-Ligand Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Subramanian	Visiting Scientist	LMB NIAMDD
Other:	P. D. Ross	Research Chemist	LMB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 34001-15 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chromatin Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Felsenfeld	Chief, Sec. on Phys. Chem.	LMB NIAMDD
OTHER:	M. Behe	Guest Worker	LMB NIAMDD
	J. McGhee	Staff Fellow	LMB NIAMDD
	J. Nickol	Staff Fellow	LMB NIAMDD
	D. Jackson	Guest Worker	LMB NIAMDD
	G. Sandeen	Chemist	LMB NIAMDD
	W. Wood	Staff Fellow	LMB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Physical Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8	6.5	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have continued our study of the structure of chromatin, the complex of proteins and DNA found in eukaryotic nuclei. Our earlier work suggested that the DNA of the nucleosome is not held in place on the surface of the particle by interactions between histones and bases. We have now shown that a considerable part of the DNA is held in place by only a few electrostatic interactions between histones and phosphodiester groups. We have also examined the 30 nm fiber formed when nucleosomes pack together in the nucleus, and determined the orientation of the nucleosomes in the fiber. We have studied the binding of histones and HMG proteins to nucleosomes, and discovered properties that may be related to higher order structure formation and to gene activation. We have developed a cell-free system for studying RNA polymerase II-dependent transcription of DNA and chromatin.

PHS-6040
(Rev. 10-76)

Project No. Z01 AM 34001-15 LMB

Project Description:

Objectives: The object of this project is to determine the structure of chromatin and the relationship of this structure to its biological role in cell differentiation.

Methods Employed: Spectrophotometry, analytical ultracentrifugation, dialysis equilibrium, and other standard techniques are used to study the binding of charged molecules to DNA, and the composition of the regions of DNA to which these molecules are bound. Standard chromatographic and electrophoretic techniques are used in purification and identification of histones. RNA polymerase is purified by classical enzymological techniques and its activity examined by membrane filtration, sucrose gradient sedimentation, or radioisotope incorporation. DNA-RNA hybridization methods are used to detect presence of specific polynucleotide sequences. Gel electrophoresis methods are used for measuring DNA and protein size, for examining nucleoprotein preparations, and for detecting chemically cross-linked proteins within chromatin. Recombinant DNA technology is employed to generate DNA fragments of defined sequence, or to insert eukaryotic DNA fragments into vectors capable of propagation in bacteria. All such work is carried out in appropriate and approved facilities under NIH guidelines. Xenopus oocytes are isolated and used for transcription studies.

Major Findings: Nucleosome structure - Our earlier work showed that nucleosome structure is not stabilized by major interactions between amino acid side chains and the bases of DNA. We therefore examined the other major class of interactions, the electrostatic attraction between the positively charged amino acids and the negatively charged phosphodiester groups of DNA. We made use of the theory of electrostatic interactions of polymers, which relates the salt dependence of interaction strength to the number of strong interactions. In the case of the nucleosome core the terminal DNA (comprising about 27% of all the DNA) can be analyzed readily. We find that for each ten base pairs (20 negative charges) there are at most 3 positive charges that interact strongly. The DNA in this region of the nuclesome is therefore held in place by a very small number of charge interactions. It is possible that all of the DNA of the nucleosome is organized in this way, and we are presently testing this hypothesis.

Higher order structure in chromatin - We have made use of electric dichroism (in collaboration with D. Rau and E. Charney, Laboratory of Chemical Physics) to study the orientation of nucleosomes in the 30 nm chromatin fiber, which is thought to consist of a solenoid in which six nucleosomes are packed in each turn. Our results show that nucleosomes must be packed with their faces nearly parallel to the long axis of the solenoid. This puts severe restrictions on possible models for the 30 nm fiber, and allows us to propose a fairly specific model. A major feature of the model is that the amino terminal tails of the histones are attached to the DNA of adjacent nucleosomes.

Histone binding to nucleosome core particles - We have extended earlier studies in other laboratories of the binding of free histones to nucleosome core particles. Detailed analysis shows that in 0.5 M NaCl, between two and three histone octomers can bind tightly to a core particle. Histones H2A and H2B alone do not bind; but histones H3 and H4 bind as a pair. The interactions are thus highly specific. The N-terminal tails of the histones can be removed with trypsin. We find that the resulting histones cannot bind to nucleosomes, although they are themselves capable of forming nucleosomes, and such trypsinized nucleosomes bind normal histones. The results are consistent with the model of higher order structure discussed above.

High mobility group (HMG) protein interactions with nucleosomes - The proteins HMG14/17 are associated with transcriptionally active genes. We have studied the binding of HMG14/17 to nucleosome cores. We find that two HMG molecules are tightly but reversibly bound to each core particle. Major sites of binding are found to occur near the ends of the core DNA. The binding to nucleosomes is tighter than to protein-free DNA. We have shown that in chicken erythroctes, HMG14/17 preferentially binds to nucleosomes containing globin genes.

Transcription of SV40 DNA by RNA polymerase II germinal vesicles - We have developed a cell-free system from the germinal vesicles of Xenopus oocytes which transcribes added DNA. We have used SV40 DNA as the template to characterize the system. Essentially all of the high molecular weight RNA product can be shown to be the result of RNA polymerase II activity. The transcripts have been characterized in terms of their size and sites of initiation; the pattern is similar to that observed for SV40 transcription in vivo. The cell-free system is also capable of coating added DNA with nucleosomes. The system is therefore a good one for studying the effects of nucleosomes on template activity under controlled conditions, and for studying the general properties of RNA polymerase II-dependent transcription.

Significance to Bio-medical Research and the Program of the Institute: Chromatin is the nucleoprotein complex that appears to contain the chemical information for cellular differentiation. Understanding of the organization of chromatin proteins on DNA and the way in which they affect transcription and other DNA functions in higher organisms will contribute to an understanding of how cells differentiate, and how they maintain this differentiated state, in which only a relatively small fraction of the total gene population of the cell is expressed. This is a central problem in cell biology.

Proposed Course of Project - We will continue the study of electrostatic interactions between DNA and chromatin in the nucleosome, in an effort to discover which interaction sites are the crucial ones. We will also continue to examine the properties of 30 nm chromatin fibers, and the way in which they are stabilized or destabilized by non-histone proteins or histone modification. The Xenopus oocyte transcriptional system will be used to examine the effects of histones on transcription, and to study transcription in vitro of cloned eukaryotic sequences.

Publications:

Felsenfeld, G., McGhee, J., Sollner-Webb, B. and Williamson, P.: The Internal Organization of the Nucleosome. ICN-UCLA Symposium on Eucaryotic Gene Regulation, 541-579, 1979.

Ginder, G.D., Wood, W.I. and Felsenfeld, G.: Isolation and Characterization of Recombinant DNA Clones Containing the Chicken Adult β-Globin Gene. J. Biol. Chem., 254: 8099-8102, 1979.

McGhee, J.D. and Ginder, G.D.: Specific DNA Methylation Sites in the Vicinity of the Chicken Beta-globin Genes. Nature, 280, 419-420, 1979.

Shindo, H. and McGhee, J.D.: ^{31}P-NMR Studies of DNA in Nucleosome Core Particles. Biopolymers 19, 523-537, 1980.

McGhee, J.D. and Felsenfeld, G.: Nucleosome Structure. Ann. Rev. Biochem., in press.

McGhee, J.D., Felsenfeld, G. and Eisenberg, H.: Nucleosome Structure and Conformational Changes. Biophysical J., in press.

McGhee, J.D. and Felsenfeld, G.: The Number of Charge-Charge Interactions Stabilizing the Ends of Nucleosome DNA. Nucleic Acids Res., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 34002-16 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Enzyme Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Davies	Chief, Sec. on Mol. Structure	LMB NIAMDD
OTHER:	G. H. Cohen	Research Chemist	LMB NIAMDD
	E. W. Silverton	Research Chemist	LMB NIAMDD
	R. Bott	Staff Fellow	LMB NIAMDD
	G. Gilliland	Staff Fellow	LMB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3-1/2	3-1/2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The refinement of the bovine proteolytic enzyme γ-chymotrypsin at 1.9 A resolution has been completed.

The aspartic protease from Rhizopus chinensis has been subjected to further high resolution data collection. A mechanism for the inhibition of the enzyme by pepstatin has been proposed. Pepstatin inhibited protein has been crystallized

The enzyme S-adenosyl methionine synthetase has been crystallized in several crystal forms that show promise of being amenable to X-ray diffraction analysis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The determination of the three-dimensional structures of enzymes with a view to understanding their mechanism of action.

Methods Employed: X-ray diffraction analysis of single crystals including heavy atom isomorphous replacement, model building, coordinate refinement in both real and reciprocal space, and computer graphics.

Major Findings: 1) A final model for γ-chymotrypsin at 1.9 A resolution has resulted from the use of the restrained least squares procedure of Drs. Wayne Hendrickson and John Konnert of the NRL. The model is well refined by conventional crystallographic criteria with R = .18 while being stereochemically acceptable. Atomic positions are estimated to be known with a precision of 0.2 Å. We have compared the model extensively with α-chymotrypsin, DIP-trypsin and elastase. The refined model includes 151 water molecules whose positions have also been compared with the water molecules of the three other structures resulting in several common sites which appear structurally equivalent. A tentative mechanism has been proposed for the conversion of the γ to the α form of the enzyme.

2) The three-dimensional structure of the aspartic protease from Rhizopus chinensis has been refined at 2.5 Å resolution to an agreement factor, R, of .279 using a restrained least squares procedure on the 10,000 data. We have proposed an amino acid sequence for the 324 residues, based on the sequence of fragments, the amino acid composition, the sequence homology between known aspartic protease sequences and the 2.5 Å electron density map. The sequence shows the same homology observed between other aspartic proteases and in three-dimensions, these regions of sequence homology tend to be clustered at or around the active site, a cleft running between the two lobes of the molecule. Data have been measured to 2 Å resolution, thereby doubling the available reflections to be used in the restrained least squares refinement procedure.

The binding of the universal aspartic protease inhibitor pepstatin has been observed in the crystal. The pepstatin is bound so that an unusual residue statine (4 amino 3-hydroxyl 6-methylheptanoic acid) is bound with the hydroxyl straddling the two active aspartic acids. The majority of pepstatin is bound as an extended chain and has allowed the identification of the P'1, P1, and P2 subsites. The binding at P1 involves a variable hydrophobic pocket which include a flexible β hairpin loop, the flap, including residues 75-85. The flap can adjust the pocket to accommodate residues such as, leucine, phenylalanine and tryptophane, which are included in the broad specificity of many aspartic proteases.

Recently we have prepared crystals of the aspartic protease and pepstatin cocrystallized in an isomorphous crystal, and also a new derivative of the aspartic protease and the peptide N acety-ala-ala-lys-vitros phe-ala-ala-NH_2, which while sharing the same space group, is not isomorphous with the crystals of the native enzyme.

3) Crystals of the enzyme S-adenosyl methionine synthetase (ATP: L-methionine S-adenosyl transferase), a 180,000 molecular weight tetramer, were grown using the vapor diffusion (having drop) method at 20°C. Two crystal forms were obtained 20 mg/ml protein solutions in .025 M Tris at pH 7.5 and 0.001 M DTT. The first has an octahedral habit and is grown in the presence of 34-35% saturated ammonium sulfate and 5% (v:v) 2-methyl-2,4-pentane diol. The crystals are tetragonal with space group $P4_12_12$ or $P4_32_12$ with unit cell dimensions a = 128.2 A and c = 172.6 A. The second form has a hexagonal bipyramid habit and is grown in the presence of 36% saturated ammonium sulfate with the addition of 0.002 M pyrophosphate and 0.01 M magnesium sulfate. The hexagonal crystals are of space group $P6_222$ or $P6_422$ with a = 128.2 A and c = 140.4 A. Both crystal forms grow to sizes ranging from 0.25 to 1.0 mm in their largest dimension and diffract to 3.5 A resolution.

Proposed Course: To refine the acid protease model to the highest resolution. To investigate the mechanism of action by inhibitor and low temperature studies.

To commence an investigation of the crystal structure of S-adenosyl methionine synthetase.

Significance to Bio-medical Research and the Program of the Institute: The acid protease is one of a large class of enzymes that include pepsin, rennin, and the cathepsins. It has sequence homology with other members of the class and the elucidation of its structure should ultimately reveal the mechanism of action of these proteins.

S-adenosyl methionine is a key molecule in biological methylation. Its formation is catalyzed by the synthetase in an unusual reaction involving ATP in which the entire tripolyphosphate chain is cleaved from the ATP and it is further degraded to PP_i and P_i before the products are released. Knowledge of the three-dimensional structure will aid understanding this mechanism.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 34003-12 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Crystal Structure Investigation of Proteins of the Immune System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Davies	Chief, Sec. on Mol. Structure	LMB NIAMDD
OTHER:	M. Navia	Staff Fellow	LMB NIAMDD
	Y. Satow	Visiting Fellow	LMB NIAMDD
	G. Gilliland	Staff Fellow	LMB NIAMDD
	G. H. Cohen	Research Chemist	LMB NIAMDD

COOPERATING UNITS (if any)

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Laboratory of Molecular Biology

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The X-ray diffraction data to 2.7 Å for McPc603 Fab have been collected and processed. The structure has been partially refined at this resolution.

X-ray diffraction data have been collected and processed to 2.5 Å resolution for the Mouse galactan-binding myeloma protein J539 Fab.

Crystals obtained from Weaver Fv, a human IgM with specific binding for pyruvylated-D-galactose, have been shown to transform to a higher symmetry when exposed to solutions of $K_2Pt(CNS)_6$. This symmetry implies that the unit cell contain either the V_H or V_L but not both.

PHS-6040

Project Description:

Objectives: A comparative investigation of the crystal structure of the Fab fragment of immunoglobulin molecules that bind strongly to several different antigens. In this way, it is hoped to map out the binding site of antibodies and to illustrate the mechanism of antigen-antibody interaction.

Methods Employed: Preparation and analysis of pure materials. Crystallization and x-ray diffraction analysis with: a) heavy atom isomorphous replacement, b) rotation function, c) molecular replacement in real space. Model building. Computer comparison of structures. Computer automated refinement of phases and atomic coordinates.

Major Findings: 1) High resolution data have been collected to 2.7 Å resolution for the mouse myeloma protein McPc 603 Fab, resulting in a total of 25,000 reflections. This represents the first successful application of oscillation camera photography and associated data processing in this laboratory. The structure is now being refined by a combination of restrained least-squares refinement coupled with an examination of difference electron density maps. The refined structure is expected to be finished this August.

2) X-ray data have been collected and processed to 2.5 Å resolution for the galactan-binding mouse myeloma Fab J539. We intend to use this to refine the previous 4.5 Å structure at the higher resolution.

3) Preliminary studies have been carried out on crystals of a protein purported to be an Fv from a human IgM with specificity for pyruvylated-D-galactose. Analysis of the change in the crystals to higher symmetry as a result of binding the heavy atom compound $K_2Pt(CNS)_6$ revealed that the symmetry of the crystals was such that the asymmetric unit could not have a molecular weight of more than 12,000. This means that the asymmetric unit cannot contain the Fv, since this has a molecular weight of 25,000. We are currently investigating whether the crystals contain V_H or V_L.

4) In collaboration with a group in the Transplantation Biology Section, Immunology Branch, NCI, we have devised a protocol for the isolation of mg quantities of miniature swine transplantation antigen. Preliminary attempts to grow crystals of this material have been successful and small crystals have been obtained. Attempts are now being made to grow these to a size suitable for x-ray diffraction analysis.

Proposed Course. 1) High resolution refinement of McPc 603 will be completed. The phosphocholine binding will be analyzed further.

2) The high resolution structure of J539 will be determined. The combining site will be compared with the model constructed from sequence alone.

3) Further attempts will be made to obtain crystals of the Fv Weaver. The current crystals will be analyzed to provide a high resolution structure of an antibody V domain.

4) A procedure will be developed to give larger crystals of SLA. These will be analyzed by x-ray diffraction methods.

Significance to Bio-medical Research and the Program of the Institute: An understanding of the molecular basis of interaction between antibody and antigen is fundamental to our understanding of the immune response. The transplantation antigens represent key proteins in modulating the immune response in ways that are not totally understood. Knowledge of their three-dimensional structure will help us to understand their function.

Publications:

Navia, M.A., Segal, D.M., Padlan, E.A., Davies, D., Rao, N., Rudikoff, S. and Potter, M.: Crystal structure of Mouse J539 galactan-binding immunoglobulin Fab at 4.5 Å resolution. Proc. Natl. Acad. Sci. USA, 76: 4071-4074, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 35000-16 LMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Chemical and Structural Investigations of Nucleic Acids and Related Substances

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHERS:	Frank B. Howard	Research Scientist	LMB	NIAMDD
	Wongil Limn	Visiting Fellow	LMB	NIAMDD
	Kunal B. Roy	Visiting Associate	LMB	NIAMDD

COOPERATING UNITS (if any)
Prof. M. Ikehara, Osaka University, Osaka, Japan
Dr. James Silverton, NHLBI
Dr. Girjesh Govil, TIFR, Bombay, India

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Organic Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 1/3	4 1/3	0

SUMMARY OF WORK (200 words or less - underline keywords)

This project has the long range objective of studying the chemistry and structure of nucleotides, polynucleotides and nucleic acids and the application of these studies to the understanding of biochemical and biological processes. Topics of current interest include: (1) structure and reactivity of polynucleotides and the relation between the two; (2) stabilization of ordered forms of nucleic acids and polynucleotides; (3) spectroscopic studies of polynucleotides: infrared, ultraviolet, and circular dichroism; (4) design and synthesis of new polynucleotides having properties required for the foregoing studies.

PHS-6040
(12-75)

Project Description:

Poly 8-Methyladenylic Acid. A Novel Polynucleotide Forming a Single-stranded Helix with Syn and Anti Conformations

We had previously synthesized poly 8-bromoadenylic acid and found that steric interference of the 8-Br substituent with the sugar ring caused all nucleotide residues of the polymer to assume a syn conformation and a hydrogen-bonded double helical structure. Since this polymer remains the only all-syn polynucleotide so far described, it is important to explore other systems to broaden our knowledge of the effect of syn conformations on polynucleotide structure. We have now synthesized and examined poly-(8MeA), in collaboration with Prof. Morio Ikehara of Osaka University.

Poly(8MeA) is novel in several respects and is totally different from both poly(8BrA) and poly(A). The neutral polymer melts sharply and cooperatively (Tm = 27^{o} ; $\sigma = 14^{o}$), unlike poly(A). The structure is, nevertheless, not double stranded since Tm is independent of salt concentration at intermediate values of ionic strength and becomes negative at high salt (cf. d Tm/ d log[Na^{+}] for poly(8BrA) = 15^{o}). Confirmation is found in the NMR spectrum of the ordered form. A somewhat broadened high resolution spectrum is observed at 19^{o}, in contrast to poly(8BrA), which has no high resolution spectrum at 19^{o} because of nuclear dipolar broadening in the rigid helix. It appears that this is the first single-stranded regular helix to be observed in aqueous solution. The polymer does not form an acid self-structure analogous to that of poly(A).

The temperature dependence of chemical shift of H2 and $8CH_3$ is quite unusual and leads to an interesting conclusion. As in many other polymers, H2 undergoes an upfield shift on polymerization due to ring current shielding of stacked bases. The magnitude of the shift is considerably larger ($\sim$ 1ppm) than those usually observed. The protons of the $8CH_3$ group, however, have a chemical shift completely independent of temperature. The conclusion seems clear that H2 is strongly stacked but 8Me is not stacked at all. Neither an all-syn nor an all-anti structure would produce this result, but CPK models indicate that alternating syn-anti conformations would have this effect. We conclude that the ordered form of poly(8MeA) has a single-stranded helical structure with regularly alternating syn and anti conformations.

Specific Structural Role of Alkali Metal Ions in the Poly(I) Helix

We had previously discovered, using infrared spectroscopy, that poly (I) helix formation exhibits a striking size-dependent specificity for the different alkali metal ions. The results were interpreted in terms of binding to carbonyl oxygens of inosine at one of two sites in the axial hole of the 4-stranded helix. Previous UV and CD studies of poly(I) have been conducted under varying conditions and have often produced conflicting results, even when apparently carried out under the same conditions. During the past year, we have studied poly(I) by UV and CD spectroscopy to

improve our understanding of dependence of helix formation on reaction conditions and to reconcile our measurements with those of other workers and with our own IR results. Though the details are too complex for brief summary, we have found that both rate and extent of helix formation depend markedly on the concentration of both poly(I) and alkali metal ion. Many of the apparent disrepancies in previous reports can be ascribed to kinetic effects and to the history of the samples, in particular whether they had been frozen before measurement. Though slow rates of reaction have so far prevented us from making equilibrium studies with K^+ and Na^+, the much faster reaction of Rb^+ has enabled us to measure binding isotherms of Rb^+ to poly(I). Both Rb^+ and Cs^+ (unlike all the other cations observed) interact with poly(I) to produce two spectroscopically distinct forms, I which is produced rapidly and II which is formed much more slowly. I is the less stable structure and can be converted to II by heating. Salt dependence curves and values of dTm/d log $[M^+]$ have been determined for all alkali metals ions and NH_4^+.

2-Amino-8-methyl-5'-adenylic acid. A Novel Conformation in the Crystal.

We have synthesized and crystallized the above nucleotide as the dihydrate, and Dr. James Silverton (NHLBI) has carried out a crystallographic analysis of this material. Though it is generally assumed in the literature that a nucleotide with a syn conformation must have C2' endo sugar puckering, this nucleotide is syn but has C3' endo puckering. The only prior report of a C3' endo structure in a syn nucleotide or polynucleotide was that of poly (8BrA) which our NMR evidence showed to have a C3' endo sugar conformation. The reason for the erroneous correlation appears to have been that previous crystallographic analyses had employed nucleosides, in which a 5'OH...N3 hydrogen bond was always present in syn conformations. We conclude that the syn - C2' endo correlation actually applies only when the hydrogen bond is present.

The $2NH_2$8Me-5'AMP structure is novel in another respect. The structure is organized by a very extensive system of hydrogen bonds into triads of nucleotides stacked upon each other and related by a three-fold screw axis. The phosphates are on the interior of the triads and the bases on the periphery. This structure bears an intriguing resemblance to the 3-stranded nucleic acid model proposed by Pauling and Corey in 1952. A major difference is that a protonated phosphate residue, required for hydrogen bonding in the Pauling and Corey model, does not actually exist in the nucleic acids, whereas the present nucleotide (a Zwitterion) is monoprotonated on phosphate and mutually hydrogen bonded by these protons to the other phosphates in the triad.

Equilibrium Binding by Continuous Ultrafiltration

The customary method of obtaining equilibrium binding information is equilibrium dialysis. This method has the disadvantages of being quite laborious when quantitative data are required and of taking several days

for equilibrium to be reached. Each point on a binding isotherm requires a separate experiment. Continuous ultrafiltration (or diafiltration) was reported a number of years ago to be much faster and more convenient, but no way was shown to obtain a binding isotherm from a wash-out experiment. In this procedure known concentions and volume of macromolecule and ligand are placed in a stirred ultrafiltration cell. As solvent is forced into the closed cell under pressure an equal volume is forced out through the membrane, collected and analysed. For complexes formed with slow kinetics, it is essential to use this method rather than previously described wash-in method (ligand in reservoir rather than cell) since equilibrium may not be obtained in the latter case. We have developed a method for obtaining binding isotherms from the wash-out procedure. In this way a single 3 hour experiment can provide data that could easily require a week to obtain by the older method. Probably the most detailed equilibrium dialysis study of monomer-polymer interaction is that of Davies and Davidson for deoxyadenosine-poly (U). We have reproduced their binding isotherm for this system, showing that the more rapid and convenient procedure of diafiltration is capable of providing the same information as the older and slower method.

Effect of $NaClO_4$ on Stoichiometry of A-U Interactions

A recent paper by Gukovskaya, Gukorsky, Chikh, and Sukhorakov (Biopolymers 19, 454 (1980) has reported that in $NaClO_4$ solutions the combining ratio of adenosine and poly (U) is changed from 1:2 (as we and others have observed in NaCl solution) to 1:1, and that 1:2 mixtures of poly (a) and poly (U) consist of both double and triple helices in $NaClO_4$. This paper has surprised most workers in the field, with opinion apparently divided as to whether it could be correct. We have reinvestigated these systems by equilibrium dialysis, diafiltration, infrared and ultraviolet spectroscopy. All methods show conclusively that the combining ratio is not changed by perchlorate ion and is 1:2 in both cases. A manuscript to correct the error is in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 35050-09-LMB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Replication, Recombination and Repair of Microbial DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Gerald Selzer	Staff Fellow	LMB	NIAMDD
	Tapan Som	Visiting Associate	LMB	NIAMDD
	Kazuo Yamaguchi	Visiting Associate	LMB	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4½	4½	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on replication of plasmid ColE1 have been continued. RNA polymerase, ribonuclease H and DNA polymerase I have been found to be essential and sufficient for initiation of DNA replication. RNA polymerase makes precursors of the primer for DNA replication and ribonuclease H cleaves the precursors at specific sites to create the primer for DNA replication that has 555 nucleatides in length. Nucleatide sequences of the regions containing the origin of replication of ColE1 related plasmids, p15A and RSF 1030, have been determined. Structural organization of the origin regions of these plasmids are quite similar but significant differences in the nucleatide sequences have also been noticed. A mechanism that regulates primer formation has been found.

Project Description:

Closed circular DNA of the plasmid ColE1 of Escherichia coli can be replicated in vitro by a soluble enzyme system. Replication starts most frequently at any of the three consecutive nucleotides (dA, dA and dC) located at a unique position on the DNA. No plasmid encoded protein is required for this replication. Initiation of replication can be demonstrated with a mixture of three highly purified E. coli enzymes: RNA polymerase, DNA polymerase I and ribonuclease H (RNase H), an enzyme that creates a 3'-OH end by endonucleolytic cleavage of RNA which is hybridized to DNA. Previously we proposed that RNA polymerase synthesizes a transcript which is processed by RNase H and then used as a primer for DNA synthesis by DNA polymerase I. To prove the hypothesis, we have constructed a plasmid (pNT7) that consists of an 812 base pair segment containing the replication origin of plasmid ColE1 and of a 1240 base pair segment containing a β-lactamase gene. Transcription of the plasmid DNA showed that the plasmid DNA has three principal sites where transcription initiates in vitro. One is located in the ColE1 segment 555 nucleotides upstream from the origin. Most transcription from this site extends past the origin; the 3' portion of some of these transcripts hybridizes spontaneously to the template DNA. When RNase H is added in the reaction, the hybridized transcripts are cleaved by the enzymes in the origin region. When DNA polymerase I is included in the reaction along with RNA polymerase and RNase H, dAMP or dCMP is added directly onto the cleaved RNA molecules, many of which have an intact 5' terminus. The addition of a deoxyribonucleotide to the cleaved RNA can be regarded as the first step of ColE1 DNA synthesis. Once it has served as a primer, the RNA is eliminated from the product by RNase H. These results support our hypothesis and revealed, for the first time, a mechanism of RNA priming in initiation of replication of double-stranded DNA.

A short (108 nucleotides) RNA (species I) is transcribed from the ColE1 segment of pNT7. This RNA inhibits formation of the primer for initiation of DNA replication by preventing hybridization of the primer transcript to the DNA template This RNA has no effect on primer formation by the related but compatible plasmids p15A, CloDF13 and RSF1030.

Nucleotide sequences around the origin of replication of P15A and RSF1030 have been determined. Structural organization of the origin regions of these plasmids are quite similar. The origin of DNA replication and the primer-promoter have been located on these plasmids.

Publications:

Itoh, T. and Tomizawa, J.: Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H and DNA polymerase I. Cold Spring Harbor Symp. Quant. Biol. 43, 409-418, (1979).

Tomizawa, J. and Selzer, G.: Initiation of DNA Synthesis in Escherichia coli. Annual Review of Biochemistry, Vol. 48, 999-1034, (1979).

Ohmori, H. and Tomizawa, J.: Nucleotide sequence of the region required for maintenance of colicin E1 plasmid. Molec. gen. Genet., 176, 161-170 (1979).

Itoh, T. and Tomizawa, J.: Formation of an RNA primer for initiation of ColE1 plasmid DNA by ribonuclease H. Proc. Natl. Acad. Sci. U.S.A., 77, 2450-2454. (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 36002-06 LMB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Novel Recombination Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. L. Rosner Research Biologist NIAMDD:LMB

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Microbial Genetics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Regulation of ISl-mediated transposition in E. coli is being studied. Of 50 compounds tested, acetate appears to enhance transposition of Tn9 (ISl-cat-ISl). UV irradiation of λ::Tn9 reduces transposability exponentially; however the reduction of ISl-cat-ISl transposition is less than ISl-λ-ISl transposition. The expectation that portions of the E. coli chromosome should behave as ISl flanked transposons has not borne out by experiment. One possibility is that resident chromosomal ISls are inactive.

PHS-6040

Project Description:

Objectives: An understanding in molecular terms of transposition, a process involving recombination between certain discrete DNA sequences (transposons) and non-homologous target chromosomes. Particular attention has been paid to the regulation of IS1-mediated transposition.

Major Findings: (1) A spot test previously developed has indicated that acetate enhances transposition of TN9 (IS1-cat-IS1) from λ::Tn9 to the E. coli chromosome. The mechanism involved is being studied.

(2) UV irradiation of λ::Tn9 results in an exponential loss of transposing ability. This is in contrast to Rec-mediated recombination which is stimulated by low doses of UV irradiation. Interestingly, the rate of UV-inactivation of IS1-cat-IS1 (Tn9) transposition is about 20% that of IS1-λ-IS1 transposition. Whether this is due to target size differences is being examined in variants having different sizes of cat relative to λ.

(3) The E. coli chromosome contains an estimated (by hybridization) 5 to 10 copies IS1. Experiments have been carried out to see if portions of the chromosome are transposable by virtue of being flanked by IS1s. Thus far, the data are negative: transposition of markers located in different parts of the chromosome occurs at least 100-1000 times less frequently than that of markers flanked by bona fide IS1s. The possibility that some IS1s are inactive is being examined.

Significance to biomedical research: The discovery of transposable elements has led to a new understanding of how chromosomal rearrangements can occur. Transposition plays an important role in evolution, gene regulation and perhaps development. Transposition of drug resistance elements is of great importance in the epidemiology of infectious diseases and chemotherapy.

Publications

Rosner, J. L. and Guyer, M. S.: Transposition of IS1-λBIO-IS1 from a bacteriophage λ derivative carrying the IS1-cat-IS1 transposon (Tn9). Mol. Gen. Genetics, 178: 111-120, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 36051-12 LMB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Genetics and structure of the oncogenic virus, SV40

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert G. Martin, Chief, Section on Microbial Genetics, LMB NIAMDD

OTHER: Roland Seif, Visiting Fellow LMB, NIAMDD
Ana Chepelinsky, Visiting Associate LMB, NIAMDD
Maria Hadjopoulos, Visiting Fellow LMB, NIAMDD
Nancy Chiu, Staff Fellow LMB, NIAMDD

COOPERATING UNITS (if any)
V. P. Setlow, Staff Fellow, NHLBI

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Laboratory of Molecular Biology

SECTION
Section on Microbial Genetics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4-1/2	4-1/2	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The 90K protein of SV40 appears to be required for the maintenance of transformation since many cell lines transformed by viral strains containing a temperature sensitive mutation in this protein (tsA mutants) are temperature sensitive for their transformed phenotype. On the other hand many cell lines are not temperature sensitive. We have examined the integration patterns of SV40 into the host in the temperature-sensitive and insensitive lines. Although some correlation between copy number and temperature sensitivity exists, there is not a simple correlation.

In separate experiments we have shown in permissively infected monkey cells that SV40 replicates > 98% from its normal origin, ~ 1% from a second origin 180° around the genome and possibly as a rare event from other sites. When tsA mutants are shifted to the nonpermissive temperature, replication from the normal sites is eliminated and initiation appears to start randomly on the SV40 genome.

Project Description:

Objective: To determine the molecular basis of transformation by SV40 using conditional lethal and viable deletion mutants.

Major Findings: (a) The pattern of integration of the SV40 genome within the cellular DNA of temperature-sensitive and temperature-insensitive hamster and rat transformants has been determined. The SV40 DNA was covalently linked to the cellular DNA in both types of transformants. In the rat cells, most temperature-sensitive transformants contained SV40 sequences integrated at a single site; most temperature-insensitive lines contained SV40 sequences integrated at 2 to 5 sites. No similar correlation was apparent in the hamster cells. In the hamster cells there appeared to be a correlation between the initial multiplicity of infection and the number of integration sites. Single copies and tandem repeats of SV40 sequences were observed in both types of transformants from rat cells. (Chepelinsky, Seif and Martin.)

(b) SV40 was found to initiate replication about 98% of the time from its normal origin and about 1% of the time from a second origin about 180° from the normal origin. When infections were carried out with tsA mutants at the nonpermissive temperature, initiation from both of these sites was markedly reduced and SV40 DNA replication now appeared to start randomly. (Setlow and Martin.)

(c) A number of revertants of temperature-sensitive and temperature-insensitive transformed cell lines have been isolated and are now being analyzed (Chou, Sief and Martin).

(d) Mutagenesis studies have been initiated with nontransformed Fisher 3T3 rat cells and transformed CHL cells (Hadjopoulos and Martin).

Publications:

Setlow, V. P., Persico-DiLauro, M., Edwards, C.A.F., and Martin, R. G.: The isolation of SV40 tsA/Deletion, Double Mutants, and the Induction of Host DNA Synthesis. Viorlogy 101, 250-260 (1980).

Seif, Roland and Martin, R.G.: Simian virus 40 small t antigen is not required for the maintenance of transformation but may act as a promoter (cocarcinogen) during establishment of transformation in resting rat cells. J. Virol. 32: 979-988 (1979).

Martin, R.G., Setlow, V. P., Chepelinsky, A.B., Seif, R., Lewis, A. M., Jr., Scher, C. D., Stiles, C. D. and Avila, J.: Roles of the tumor antigens in transformation by SV40. Cold Spr. Harb. Quant. Biol., in press.

Chepelinsky, A. B., Seif, R. and Martin, R.G.: Integration of the simian virus 40 genome into cellular DNA in temperature-sensitive (N) and temperature-insensitive (A) transformants of 3T3 rat and Chinese hamster lung cells. J. Virol., in press.

Martin, R. G. and Setlow, V. P.: The initiation of SV40 DNA synthesis is not unique to the replication origin. Cell, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 36101-06 LMB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Energy conversion in biology, especially muscle contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Terrell L. Hill	Chief, Section on Theoretical Molecular Biology, LMB NIAMDD
Other:	Yi-der Chen	Research Chemist, LMB NIAMDD

COOPERATING UNITS (if any)

Evan Eisenberg	Heart, LB
Leonard Stein	Heart, LB
J. M. Chalovich	Heart, LB

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Laboratory of Molecular Biology

SECTION
Theoretical Molecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Four different topics have been studied under this project:

(a) (Eisenberg, Hill, Chen). Isometric transient and steady contraction properties have been calculated from a self-consistent theoretical model of muscle contraction. Agreement with experiment is very good. Recent biochemical results, however, compel us to extend the model to eight states (four attached, four unattached). We are presently engaged in adjustment of model parameters to obtain the best possible fit with experimental data. The unique feature of this work is the bridge we are building between muscle biochemistry and muscle biophysics.

(b) (Hill, Eisenberg, Chalovich). In preparation for future extension of the above model to the regulation of muscle contraction by Ca^{++}, we have developed a model for the cooperative steady-state ATPase activity of myosin subfragment-1 on actin-troponin-tropomyosin. Experiments (by Chalovich and Eisenberg) are in progress that will be compared with the theory.

(c) (Hill). A much more detailed account has been published on the thermodynamic and kinetic interrelationships between the four main membrane systems of mitochondrial oxidative phosphorylation: respiratory chain; reverse ATPase, ADP-ATP exchange, and H^+, P_i symport. Also this treatment has been extended in two ways: the tight coupling assumed between the two subforces and subfluxes for each of the four systems has been relaxed to allow for the experimentally indicated incomplete coupling (inefficiency); and the four systems in the model have been extended to eight, to allow for H^+ leak, Ca^{++} leak, Ca^{++}-H^+ exchange; and ATP consumption in various processes within the mitochondrial matrix.

(d) (Hill, Stein). A comprehensive theoretical study has been made of the cooperative properties of a lattice of interacting three-state enzyme molecules. Also we have examined the limits of the validity of the assumption, in biochemical kinetics, that the discrete states of a biochemical cycle, operating at steady state, are themselves separately in an internal equilibrium among substates.

Publications

Eisenberg, E., Hill, T. L. and Chen, Y.: Cross-bridge model of muscle contraction; quantitative analysis. Biophysical J. 29, 195-227, 1980.

Hill, T. L., Eisenberg, E. and Chalovich, J. M.: Theoretical models for cooperative steady-state ATPase activity of myosin subfragment-1 on regulated actin. Proc. Natl. Acad. Sci. USA, in press.

Hill, T. L.: Effective coupling of four independent membrane systems in steady-state oxidative phosphorylation. Cell Biophys., in press.

Hill, T. L.: Steady-state properties of coupled systems in mitochondrial oxidative phosphorylation. Proc. Natl. Acad. Sci. USA, in press.

Hill, T. L. and Stein, L.: Properties of some three-state, steady-state Ising systems, according to the Bragg-Williams approximation. Proc. Natl. Acad. Sci. USA 77: 693-697, 1980.

Hill, T. L.: Examination of the conventional assumption of internal equilibrium within the states of a biochemical cycle. Proc. Natl. Acad. Sci. USA 77: 205-209, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 36102-09 LMB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Statistical thermodynamics of protein and polynucleotide systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Terrell L. Hill, Chief, Section on Theoretical Molecular Biology, LMB, NIAMDD

OTHER: Yi-der Chen Research Chemist, LMB, NIAMDD
Takashi Tsuchiya Visiting Associate, LMB, NIAMDD

COOPERATING UNITS (if any)

Evan Eisenberg, Heart, LB
Lois Greene, Heart, LB

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Theoretical Molecula Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Four separate topics have been investigated here:

(a) (Hill, Eisenberg). The equilibrium binding constants of the myosin fragments S-1 and HMM onto an actin filament have been calculated, semi-quantitatively, from statistical mechanical first principles. The relations between one-headed and two-headed binding are especially interesting. Agreement with experiment is quite satisfactory.

(b) (Hill, Eisenberg, Greene). The experimental data of Greene and Eisenberg on the cooperative equilibrium binding of S-1-ADP onto actin-troponin-tropomyosin, in the presence and absence of Ca^{++}, have been matched very well by a theoretical treatment that is a rather complicated extension of the well-known two-state, linear Ising model. The experiments and the theory are being extended to allow for different salt concentrations and for different nucleotides bound to the S-1.

PHS-6040

(c) (Hill). Steady-state head-to-tail polymerization of actin or of microtubules require a flux of ATP or of GTP, respectively. in this work, an explicit kinetic model was used to examine the bioenergetic aspects of this process. Also investigated, for an important special case, was the steady-state polymer length distribution. The distribution found is of the same form as found experimentally.

(d) (Chen). In general, the structure of a covalently-closed circular DNA in solution depends on the bending and torsional stiffness of the double helix of the DNA molecule. The resistance of DNA to bending can be deduced from the experimentally determined DNA persistence length. In contrast, the torsional stiffness remains obscure. This is because the torisonal stiffness does not affect any properties of linear DNA molecules. It has been reported that theoretical studies of fluctuations of writhing numbers of model ring polymers could be used to estimate the torsional stiffness of DNA molecules. A necessary step in these studies is to generate a large number of ring polymers. In this current study, we have devised an efficient method of generating ring polymers (on lattice or off lattice) based on the "dimerization" method of Alexandrowicz. The torsional stiffness of DNA molecules will be studied using the ring polymers generated by this method.

Publications

Hill, T. L. and Eisenberg, E.: Theoretical considerations in the equilibrium binding of myosin fragments on F-actin. Biophysical Chemistry 11: 271-281, 1980.

Hill, T. L., Eisenberg, E. and Greene, L.: A theoretical model for the cooperative equilibrium binding of myosin subfragment-one to the actin-troponin-tropomyosin complex. Proc. Natl. Acad. Sci. USA, in press.

Hill, T. L.: Bioenergetic aspects and polymer length distribution in steady-state head-to-tail polymerization of actin or microtubules. Proc. Natl. Acad. Sci. USA, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 36103-09 LMB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Ion transport across the nerve axon membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Yi-der Chen Research Chemist LMB, NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Theoretical Molecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Recently, Fishman and his colleagues have found a low frequency feature in the admittance (or impedance) of squid axon membranes which cannot be accounted for by the usual Hodgkin-Huxley formalism. Two physical processes which slightly modify the usual HH formalism have been proposed to explain this low frequency feature by Fishman and his colleagues. One process involves the addition of an inactivation to the HH K^+ kinetics. The other involves the K^+ accumulation outside the axon membrane. In our work, we have shown that the experimental low-frequency feature can be simulated easily with a "non-equilibrium" channel model. This model differs from the original Hodgkin-Huxley formalism and the modified models mentioned above in that this model requires an energy source to maintain a nonequilibrium steady state. In squid axons, the electrochemical gradients of K^+ and Na^+ may serve as the energy source.

PHS-6040

Reference:

Chen, Y.: On the admittance of membranes associated with channel conduction. Application to channels at nonequilibrium steady-state. J. Theoret. Biol. 81: 633-644, 1979.

ANNUAL REPORT OF THE LABORATORY OF BIOORGANIC CHEMISTRY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

SECTION ON OXIDATION MECHANISMS

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

Previous annual reports from this Section have described a systematic approach to the study of the cytotoxicity, mutagenicity, and carcinogenicity of the ubiquitous environmental carcinogen benzo[a]pyrene (BP). Briefly these studies have consisted of i) synthesis of as many known and potential oxidative metabolites as was possible, ii) study of the metabolism of the hydrocarbon with these authentic standards in hand, iii) testing of these compounds for cytotoxic and mutagenic activity with bacterial and mammalian cells both in the presence and in the absence of added drug metabolizing systems such as cytochrome P-450 and epoxide hydrolase, and iv) evaluation of the carcinogenicity of these synthetic standards in several animal models. These studies provided evidence which indicated that (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene is the most potent carcinogenic metabolite of benzo[a]pyrene. With the first identification of an ultimate carcinogenic form for any member of the polycyclic aromatic hydrocarbon class of carcinogens, we formulated the "bay-region" theory which predicts that diol epoxides that have the epoxide group in the bay-region of the hydrocarbon will be the most chemically reactive and presumably biologically active. To test the predictions of the theory, we have continued studies on the hydrocarbons benzo[a]anthracene, chrysene, dibenzo[a,h]anthracene, and 3-methylcholanthrene, and have initiated new studies on benzo[e]pyrene, cyclopenta[c,d]pyrene, benzo[c]phenanthrene, and dibenzopyrenes.

Benzo[a]pyrene (BP). (+)-7β,8α-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro BP had been described in the previous report as the most active ultimate carcinogenic metabolite of BP known on mouse skin and in the newborn mouse tumor model. Current studies have explored the ability of BP and 13 of its derivatives to induce fibrosarcomas after subcutaneous injection into C3H/fCum mice. This is an important tumor model since it had been used so extensively to test hydrocarbons for tumorigenic activity in the past. It is, however, an unusual model since it often fails to detect proximate and ultimate carcinogens. For example, the proximate carcinogen (+)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene had very low activity when injected in trioctanoin. This activity was increased 10- to 15-fold when the dihydrodiol was injected in DMSO or when the diacetate of the dihydrodiol was injected. The results are suggestive that unusual transport problems may be associated with the more polar oxygenated metabolites in this tumor model. Similarly, the above diol epoxide and 2-hydroxy BP, both potent carcinogens, were very weak in this tumor model. These results demonstrate the importance of evaluating the carcinogenic potential of chemicals in more than one tumor system. In the newborn mouse, 2-hydroxy BP caused about 4-fold more pulmonary

tumors than BP but was equipotent to BP on skin. In none of these three tumor models has 6-hydroxy BP shown significant tumorigenic activity. This is a critical finding in light of the numerous studies which had attempted to show that this reactive molecule is an ultimate carcinogen.

In order to study the biological effects of (+)- and (-)-benzo[a]-pyrene 4,5-oxide, a synthesis of these molecules has been developed based on the resolution of (±)-cis-4,5-dihydroxy-4,5-dihydrobenzo[a]pyrene. The (-) enantiomer of benzo[a]pyrene 4,5-oxide was 1.5- to 5.5-fold more mutagenic than the (+) enantiomer in strains TA 98, TA 100, TA 1537, and TA 1538 of *Salmonella typhimurium* and in Chinese hamster V79 cells. In studies with V79 cells, the (-) enantiomer of benzo[a]pyrene 4,5-oxide was also more cytotoxic than the (+) enantiomer. When mixtures of the enantiomers were studied in V79 cells, synergistic cytotoxic and mutagenic responses were observed. The greatest cytotoxic and mutagenic effects occurred with a 3:1 mixture of the (-) and (+) enantiomers of benzo[a]pyrene 4,5-oxide, respectively.

Kinetic studies of the bay-region 7,8-diol-9,10-epoxides of BP have indicated that these molecules are quite sensitive to general acid catalyzed hydrolysis. Perhaps the most important conclusion that can be drawn from our present results is that detoxification in the cell might occur by their reactions with noncritical cellular agents by a mechanism that involves general acid catalysis, as opposed to a mechanism in which these agents act as nucleophilic scavengers. However, binding of these diol epoxides to critical cellular reagents such as DNA might also be facilitated by this same type of general acid catalysis. Thus, comparisons of second-order rate constants for the attack of nucleophiles with the solvolytic rate constants may be of limited value in predicting the biological activity of the diol epoxides. In summary, present results indicate that water is probably the cell's best protective agent against the deleterious effects of these reactive diol epoxides. Enzymic pathways such as those involving epoxide hydrolase and GSH-transferase are ineffective in the detoxication of diol epoxides.

Benzo[a]anthracene (BA) and Dibenzo[a,h]anthracene (DBA). Although DBA is known to be a more potent carcinogen than BA, the bay-region diol epoxides of BA are expected to be more reactive than those of DBA. In the previous report, we had speculated that part of the reason for this was due to the very low conversion of BA into its 3,4-dihydrodiol - the immediate metabolic precursor of the diol epoxide. Current studies of the metabolism of DBA by liver microsomes from control and induced rats as well as by a highly purified and reconstituted system have shown that its highly tumorigenic 3,4-dihydrodiol constitutes 24-28% of the total metabolism of the hydrocarbon. This is the most extensive known conversion of a hydrocarbon into a proximate dihydrodiol carcinogen. Further metabolism of the 3,4-dihydrodiol occurred to form 3,4-diol-1,2-epoxides which were detected as their tetraol solvolysis products. These same products could be identified as metabolites when DBA was used as the substrate. Thus, DBA undergoes rather facile conversion into its bay-region diol epoxides. This facile metabolism of DBA combined with the low conversion of BA into its 3,4-dihydrodiol is thought to be a major determinant in the relative activity of the two hydrocarbons.

Through a combination of chemical degradation and spectral techniques, it has not been possible to assign absolute configurations to the 3,4-, 5,6-, 8,9-, and 10,11-dihydrodiols of BA. The predominant enantiomers of the metabolically formed dihydrodiols have [R,R] absolute configuration. Planned metabolism studies of the optically active benzo-ring dihydrodiols will seek to determine if the cytochromes P-450 selectively produce diol epoxide diastereomers in which the benzylic hydroxyl group is trans to the epoxide oxygen (diol epoxide-2 series), as was the case for the BP 7,8-dihydrodiol. Since studies in the newborn mouse have shown that the (-)-BA 3,4-dihydrodiol and the BA 3,4-diol-1,2-epoxide-2 are the most tumorigenic stereoisomers, it seems probable that the most active diol epoxide of BA will directly superimpose on that from BP. Thus of four possible stereoisomers, the most tumorigenic isomer is that which is metabolically predominant.

Benzo[e]pyrene (B[e]P). Tumor studies both on mouse skin and in newborn mice have shown that B[e]P as well as its 4,5- and 9,10-dihydrodiols are practically inactive as carcinogens. Several approaches were undertaken in order to explain the lack of activity of the parent hydrocarbon. The first of these was a renewed attempt to prepare the diastereomeric bay-region 9,10-diol-11,12-epoxides. Earlier studies had shown that mixtures were formed which could not be separated without concomitant decomposition. A major advance was made with the finding that high performance liquid chromatography could be used to separate the mixture. Bay-region diol epoxides of several hydrocarbons were found to chromatograph intact on Du Pont SIL columns eluted with mixtures of tetrahydrofuran and hexane. Once the B[e]P diol epoxides were obtained in pure form, nmr spectroscopy established that both diastereomers exist predominantly in the conformation with quasi-diaxial hydroxyl groups. This is the first example of a diol epoxide-2 diastereomer in which the hydroxyl groups are quasi-diaxial. Both isomers were found to be very weak mutagens toward bacterial and mammalian cells - presumably because of the quasi-diaxial hydroxyl groups since the 9,10-epoxide of 9,10,11,12-tetrahydro B[e]P is highly mutagenic. Metabolic factors also contribute to the lack of tumorigenicity of B[e]P. The possible proximate carcinogenic metabolite, B[e]P 9,10-dihydrodiol, was formed by liver microsomes but constituted less than 1% of the total metabolites. Furthermore, no more than trace amounts of the bay-region diol epoxides were formed from this dihydrodiol, presumably because it has quasi-diaxial hydroxyl groups.

Phenanthrene and Chrysene. Studies on the racemic dihydrodiols and bay-region diol epoxides of chrysene and phenanthrene are now essentially complete. The bay-region 1,2-diol-3,4-epoxides of chrysene were as much as 40-fold more mutagenic toward mammalian and bacterial cells than was chrysene K-region 5,6-oxide. The phenanthrene diol epoxides were much less mutagenic than the corresponding chrysene diol epoxides. In initiation-promotion experiments on mouse skin, phenanthrene and its three metabolically possible trans dihydrodiols were found to be practically inactive as tumorigenic agents. In the newborn mouse tumor model, (+)-1β, 2α-dihydroxy-3α,4α-epoxy-1,2,3,4-tetrahydrochrysene (diol epoxide-2) was the most potent chrysene derivative tested. It induced pulmonary tumors in 98% of the mice, with an average of 15.9 tumors/mouse. (+)-1β,2α-Dihydroxy-3β,4β-

epoxy-1,2,3,4-tetrahydrochrysene (diol epoxide-1), had little if any tumorigenic activity at the dose tested. trans-1,2-Dihydroxy-1,2-dihydrochrysene and 1,2-dihydrochrysene, the immediate metabolic precursors of a bay-region diol-epoxide and a bay-region tetrahydroepoxide, respectively, were the next most active chrysene derivatives tested. Phenanthrene and its derivatives were less tumorigenic than chrysene and its derivatives. Phenanthrene, 1,2-dihydrophenanthrene, and the two diastereomeric bay-region diol epoxides of phenanthrene had little or no tumorigenic activity, but the bay-region tetrahydroepoxide, 3,4-epoxy-1,2,3,4-tetrahydrophenanthrene, had some activity.

3-Methylcholanthrene (MC). In the previous report, we had described the isolation of an MC 9,10-dihydrodiol which was also hydroxylated on the C-1 methylene group. This dihydrodiol with a bay-region double bond was readily activated by liver microsomal enzymes to potent mutagens. In initiation-promotion experiments on mouse skin, comparisons of the percentage of mice with tumors and the average number of tumors per mouse indicated that 3-methylcholanthrene, 2-hydroxy-3-methylcholanthrene, 3-methylcholanthrene-2-one, and a 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene diastereomer were approximately equipotent as tumor initiators. In the newborn mouse tumor model, 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene was the most tumorigenic compound tested. 3-Methylcholanthrene and 3-methylcholanthrene-2-one were the next most active compounds, and they had approximately one-half to one-third of the activity of 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene when the data were expressed as pulmonary tumors per mouse. 1,9,10-Trihydroxy-9,10-dihydro-3-methylcholanthrene also produced hepatic tumors. 3-Methylcholanthrene and 2-hydroxy-3-methycholanthrene had one-fourth to one-tenth the activity of 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene in the liver. The other 3-methylcholanthrene metabolites were essentially inactive in producing hepatic tumors. The high tumorigenic activity of 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene on mouse skin and in newborn mice provides evidence for bay-region activation of 3-methylcholanthrene to an ultimate carcinogen. The data also indicate that other metabolites of 3-methylcholanthrene may play a role in the carcinogenicity of this polycyclic aromatic hydrocarbon.

Cyclopentapyrene (CPP). Substantial interest has developed in the hydrocarbon CPP because of its wide distribution in the environment and the ease with which it is metabolically activated to mutagens. Only one tumor study had been done which suggested that the hydrocarbon might be tumorigenic. Since CPP has no bay-region, some authors claimed it was an exception to the bay-region theory. When an appropriate set of compounds were tested, it was established that the metabolism-induced mutagenicity of CPP was in no way unique or unusually high. Benzo[a]pyrene was found to be at least 20-fold more active as a tumor-initiator on mouse skin than was CPP, which had activity comparable to BA. CPP is significantly less active than chrysene. Thus, contrary to inferences made from its high mutagenic activity, CPP is a very weak tumor-initiator on mouse skin. The substantial effort which NCI is putting into this compound may not be justified.

Dihydrodiol Synthesis. In the past year, the Section has entered into the study of three new hydrocarbons to test the bay-region theory. Two of

these are the potent carcinogens dibenzo[a,h] and [a,i]pyrene. Fairly direct methods have been utilized to synthesize the key dihydrodiols with bay-region double bonds for each of these, and the dihydrodiols have been further transformed into their bay-region diol epoxides in the isomer-2 series. Thus far, several methods have failed to yield their isomer-1 congeners. The other hydrocarbon studied was the very weak carcinogen benzo[c]phenanthrene. The study of this compound was undertaken because it has a unique, highly hindered bay-region between C-1 and C-12. Because of the high degree of steric hindrance in this area, it was impossible to synthesize its bay-region 1,2-dihydrodiol by direct methods. A direct autoxidation route did, however, prove successful. The 3,4- and 5,6-dihydrodiols were obtained by direct methods.

Epoxide Hydrolase. Human liver microsomal expoxide hydrolase has been highly purified to a specific activity (570 to 620 nmol/min/mg of protein) comparable to that of the rat enzyme using styrene oxide as substrate. Like the purified rat liver microsomal epoxide hydrolase, the human enzyme has a minimum molecular weight of 49,000 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and exhibits broad substrate specificity toward a variety of alkene and arene oxides. Despite these similarities, the human and rat enzymes are different proteins as judged by their immunochemical properties as well as their relative catalytic activities toward certain substrates.

Highly purified rat hepatic microsomal epoxide hydrolase, which had been purified in the presence of proteolytic enzyme inhibitors, was subjected to carboxypeptidase Y digestion, automated Edman degradation, and carbohydrate analysis. Carboxypeptidase Y digestion resulted in the near stoichiometric release of leucine, the COOH-terminal amino acid. Automated Edman degradation permitted the identification of the first 20 amino acid residues of epoxide hydrolase. Methionine was identified as the NH_2-terminal residue. The NH_2-terminal region of epoxide hydrolase is similar in hydrophobicity to the NH_2-terminal precursor segments of several secretory proteins and the NH_2-terminal regions of several microsomal cytochromes P-450. Carbohydrate analyses of the enzyme revealed the presence of 0.5 to 1.0 mol of mannose/50,000 g of protein. These results provide evidence for the presence of a single polypeptide chain in our purified enzyme preparations and suggest that there may be only one enzymic form of epoxide hydrolase in microsomes from phenobarbital-treated rats.

We have developed a rapid, continuous, and highly sensitive fluorescence assay for the measurement of epoxide hydrolase activity. The method is based on the large differences between the fluorescence spectra of certain K-region arene oxides and their corresponding trans-dihydrodiols. Enzymatic hydration of K-region arene oxides of phenanthrene, pyrene, benzo[a]pyrene, and 7,12-dimethylbenzo[a]anthracene was studied. The assay was most sensitive with benzo[a]pyrene-4,5-oxide as substrate. With 10 μM benzo[a]pyrene-4,5-oxide, enzymatic rates of 30 pmol of dihydrodiol/min/mg of protein are three to five times those of the blank without enzyme. The fluorometric method described has been used to study site-directed inhibitors of epoxide hydrolase and the stereoselective hydration of racemic arene oxides.

The studies have been conducted primarily by Drs. D. Jerina, H. Yagi, D. R. Thakker, R. N. Armstrong, M. Nordqvist, R. E. Lehr, H. D. Mah, S. Turujman, G. C. DeBoise, and Y. Ittah of this laboratory. A portion of the studies was done in collaboration with Drs. A. H. Conney, W. Levin, and A. W. Wood at Hoffman-La Roche, with Dr. D. R. Boyd at Queen's University, and with Dr. D. L. Whalen at The University of Maryland.

SECTION ON PHARMACODYNAMICS

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

Skin extracts of frogs have yielded further supplies of batrachotoxins, pumiliotoxin A, B and C, histrionicotoxins and gephyrotoxin for pharmacological investigation. Various additional alkaloids have been isolated. These include: 1) a hydroxybatrachotoxin, whose structure is under investigation, 2) three more histrionicotoxins ($C_{19}H_{25}NO$, $C_{17}H_{25}NO$, and $C_{19}H_{27}NO$), whose structures have been elucidated by nuclear magnetic resonance spectroscopy, 3) isomeric allopumiliotoxins ($C_{19}H_{33}NO_3$) which differ only in the stereochemistry of the 7-hydroxy substituent characteristic of this subclass of pumiliotoxin-A alkaloids, 4) an alkaloid ($C_{16}H_{29}NO_2$) from a bufonid toad which appears to differ from dendrobatid pumiliotoxin-A alkaloids only in the position of side chain hydroxyl group. Mass spectral analysis defines the structure but not the stereoconfiguration of the compound, 5) a tricyclic monohydroxy alkaloid ($C_{10}H_{29}NO$) which on mass spectral analysis affords a base peak ($C_7H_{13}N$) with an odd number of hydrogens, an unusual phenomenon for an alkaloid. A number of dendrobatid alkaloids of this class have now been identified, 6) an alkaloid ($C_{15}H_{25}N$) which was previously proposed to be a member of the pumiliotoxin-C class of decahydroquinolines, was shown by magnetic resonance spectroscopy to represent another class of alkaloids, probably a deoxyhistrionicotoxin with 2-allyl and 7-vinyl substituents. A trace indolizidine alkaloid ($C_{15}H_{29}N$), present in certain dendrobatid frogs, was compared with a synthetic 2-butyl-5-propylindolizidine, synthesized by Dr. MacDonald, Vanderbilt University. Gas chromatographic mass spectral analysis indicated that the dendrobatid alkaloid has the same gross structure as the synthetic material but differs in stereochemistry. Analysis of aldehydes obtained as periodate cleavage products from pumiliotoxin-B by proton magnetic resonance spectroscopy indicate that the double bond has the major substituents trans rather than cis as previously suggested for this alkaloid. Systems for separation of dendrobatid alkaloids by high pressure liquid chromatography have been developed. Such systems should prove useful for the small scale separation and isolation of such compounds.

Further analysis of the analgesic compound present in trace amounts in the frog Dendrobates tricolor indicate that pyrolysis of this compound occurs during gas chromatography or mass spectrometry to yield a relatively nonpolar pyridoindolizidine with an empirical formula of $C_{10}H_{11}N_2OCl$ and that the parent analgesic alkaloid is more complex and polar in nature, perhaps related in structure to the pumiliotoxin-A class of alkaloids.

Alkaloids were analyzed in six further species of dendrobatid frogs, three of which represent previously undescribed species. No new classes of compounds were detected, but the data permit further evolutionary relation-

ships within the family Dendrobatidae to be discerned. Variation of alkaloid profiles in populations of frogs and in individual frogs has, however, complicated application of such data for taxonomic purposes. It also appears that mechanisms regulating the biosynthesis of the diverse complex of dendrobatid alkaloids are controlled by many factors, both genetic and environmental. Certain species such as Phyllobates terribilis, when raised in captivity do not produce detectable levels of alkaloids. Wild individuals of this species contain high levels of batrachotoxin and are the most toxic of the dendrobatid frogs. The captive-raised frogs, although not producing detectable levels of batrachotoxin remain insensitive to its action on sodium channels in nerve and muscle. The importance of channel regulatory sites at which these alkaloids interact is suggested in that this site appears to have been only minimally altered in Phyllobates terribilis so as to prevent interaction with batrachotoxin, while remaining sensitive to other sodium conductance activators (veratridine, grayanotoxin) to which the frogs are not exposed. Offspring of the brightly colored alkaloid-producing Dendrobates tricolor and the dull colored alkaloid-free Dendrobates anthonyi produce no detectable alkaloids, nor do subsequent backcross offspring. Stress appears to increase levels of alkaloids considerably in captive Dendrobates auratus, suggesting that the role of stress in triggering alkaloid production in captive-reared dendrobatid frogs should be investigated.

Pumiliotoxins of the A class, in particular pumiliotoxin-B, potentiate and prolong stimulus-elicited contractures of striated muscle. In frog sciatic nerve-sartorius muscle the potentiation is followed by a stimulus-dependent depression of twitch. The presence of high concentrations of extracellular calcium prevent depression of muscle twitch. The presence of pumiliotoxin-B in nerve-muscle preparations causes single nerve stimuli to trigger repetitive endplate potentials which often summate to give an action potential and muscle twitch. Pumiliotoxin-B has no apparent effect on sodium or potassium conductances, but would appear to enhance excitation-contraction and excitation-secretion coupling by facilitating mobilization of calcium ions in nerve and muscle.

Pumiliotoxin-C and analogs inhibit neuromuscular transmission in frog sciatic nerve-sartorius muscle and rat phrenic nerve-diaphragm muscle. The most potent analog PTX-C_{II} (2,5-dipropyl-cis-decahydroquinoline) at 40 μM blocks indirectly elicited muscle twitch in 45 min in frog muscle. It has no effect on muscle membrane potential. PTX-C_{II} markedly depresses the amplitude and frequency of miniature endplate potentials and amplitude of endplate potentials as well as completely blocking extrajunctional sensitivity to iontophoretic acetylcholine in chronically denervated rat soleus muscle. The blockade of neuromuscular transmission by PTX-C_{II} is readily reversible. PTX-C_{II} is equipotent to D-tubocurarine with respect to inhibition of binding of radioactive α-bungarotoxin to acetylcholine receptors in Torpedo electroplax membranes. These decahydroquinoline alkaloids appear to represent a new class of nicotinic antagonists which prevent neuromuscular transmission by blockade of acetylcholine receptors.

Batrachotoxin, veratridine and grayanotoxin depolarize neuronal elements in a microsac preparation from guinea pig cerebral cortex. Tetrodotoxin blocks the depolarization while the polypeptide anenome toxin potentiates

the response to batrachotoxin, veratridine and grayanotoxin. An additional potassium-sensitive depolarization in these microsac preparations probably represents the response of glial elements. Membrane potential was determined from uptake of radioactive triphenylmethylphosphonium ion.

Batrachotoxin-A 20-α-benzoate (BTX-B) is equipotent to batrachotoxin itself in toxicity and in depolarizing nerve muscle preparations. This was unexpected since previous data had suggested the importance of a 20-α-pyrrole-3-carboxylate moiety as a prime determinant of the biological activity of batrachotoxin class of compounds. Radioactive BTX-B binds with high affinity to sites in mouse brain membranes. Specific binding represents only about ten percent of total binding at 150 nM [^{3}H]BTX-B, but the high reproducibility of a centrifugal assay permits investigation of this specific binding component which appears to have a density in brain similar to that of saxitoxin and tetrodotoxin-binding sites. Specific binding of [^{3}H]BTX-B is antagonized by compounds thought to interact with batrachotoxin-sensitive regulatory site on voltage-sensitive sodium channels with the expected order of potency: batrachotoxin > veratridine > grayanotoxin. Batrachotoxin-A also appears to displace [^{3}H]BTX-A from specific binding sites which is unexpected since this analog is not a potent activator of sodium channels. Tetrodotoxin, scorpion toxin and anenome toxin have no apparent effect on binding of [^{3}H]BTX-B. BTX-B appears to represent a new radioactive ligand for the investigation of sodium channels.

Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

Specific antibodies to rat liver catechol-O-methyltransferase (COMT) are continuing to be utilized to determine the localization of COMT at the cellular and subcellular level; to measure the rate of synthesis and degradation of the enzyme; and to measure and identify COMT in various hybrid cell lines.

An extensive study of the localization of COMT in the central nervous system of the rat is in the final stages of completion. The primary areas of localization include the oligodendroglia, astroglia and microglia; the leptomeninges, choroid plexuses, the ventricular ependyma, the tanycyte ependyma and the circumventricular organs. The major conclusions arising from this study are: 1) a non-neuronal localization of COMT; 2) oligodendrocytes and astrocytes in the densely ennervated fields of catecholaminergic terminals could provide a physiological mechanism for the restriction of transmitter migration; 3) the high concentrations of COMT in ventricular ependyma, choroid plexuses and circumventricular organs could control the accessibility of physiologically active catechols at the interfaces of brain and cerebrospinal fluid, blood and CSF, and blood and the brain parenchyma. However the metabolism of catecholamines in the immediate local of their release from axonal varicosities or terminal boutons does not appear to be a major function of COMT. Instead the localization is consistent with a) the regional restriction of the effects of catecholaminergic systems of diffuse ennervation, b) the restriction of areas in which passage of native catecholamines in which the passage between the brain parenchyma and CSF is possible, and c) separation of the effects of peripheral, circulating catecholamines from the effects of centrally derived catecholamines.

Immunocytochemical localization of COMT has been studied in the pituitary gland of rat. Intense COMT immunofluorescence is observed in pituicytes of the neural lobe, cells of the pars intermedia are negative and only the folliculo-stellate cells of the pars distalis contain COMT.

Localization studies on the adrenal gland revealed that COMT is almost exclusively in the zona glomerulosa. The adrenal medulla is devoid of COMT except in connective tissue elements.

An initial study of COMT in the eye of the adult rat reveals specific immunofluorescence of glia in the ganglion cell layer of the retina and in the choroid cells. The ciliary body is intensely fluorescent and similar in appearance to the choroid plexuses of rat brain. It is postulated that the role of COMT in this blood-aqueous barrier is similar to that of the choroid plexus at the blood-CSF barrier.

Studies have been initiated to determine the localization of COMT in the spinal cord and the peripheral nervous system. Preliminary experiments indicate the presence of COMT in Schwann cells ensheathing axons and in "glial-like" cells in the superior cervical ganglion.

The exploration of the localization of COMT in the cardiovascular system has been expanded to include both arterial and venous elements from the aorta and vena cava to capillaries. The present studies indicate that COMT is present exclusively in endothelial cells and not in smooth muscle cells. Subcellular studies utilizing the peroxidase-antiperoxidase method at present appear to confirm this localization. Because of the variance of this localization (that is the apparent absence of COMT in vascular smooth muscles), with conclusions based on biochemical and pharmacological findings this observation is being extended to include a study of COMT localization in a variety of cultured smooth muscle systems.

An initial study of the changes in the cellular localization of COMT in rat uterus during the course of pregnancy has been completed. Briefly, the enzyme is limited to epithelial cells of the uterus up to day 6. At day 7 with the implantation of the blastocyst cells containing COMT appear in the decidua in a characteristic geometric configuration on the antimesometrial side of the uterus. The appearance of COMT, as determined cytochemically, on implantation of the blastocyst, is being confirmed biochemically. The localization of COMT, estrogen hydroxylase and the tissue levels of 2-hydroxy-estradiol are being measured in a series of timed pregnancies in rat. Preliminary evidence indicates that the cytochemical changes in COMT are similar to those determined biochemically.

The immunochemical demonstration that COMT is markedly elevated in malignant breast tumors has been confirmed by direct biochemical assay of COMT, both in man and in spontaneous tumors in rat and mouse. A preliminary examination of both non-lactating rat and mouse has clearly demonstrated that the level of COMT in breast tissue increases during lactation, although the observed increase is not as dramatic as the increase seen in malignant breast tissue. The localization in ductal cells, is similar in the lactating rodent and in tumor tissue. Several rodent and human metastatic tumors of mammary origin have been examined and shown to maintain the high levels of COMT seen in the primary tumor.

Rabbit anti-rat COMT has been used to measure COMT levels by immunotitration in a comparison of the levels of COMT during the development of two genetically different rat strains. One strain Fischer-344 rats have low COMT activity which is inherited in an autosomal recessive fashion. The Fischer-344 strain was compared with the Wistar-Furth strain. The results of this study indicate that regulatory rather than temporal gene effects are responsible for the genetic differences in COMT levels seen in these two rat strains. This study has been extended to include the spontaneously hypertensive Aoki-strain rat. A comparison of the turnover rate of COMT in the liver, kidney and heart in three genetically different rat strains is in progress. Preliminary results indicate that regulatory gene effects are again responsible for the genetic differences.

Investigation of differences in the agonist specificities for the ring fluorinated derivatives of norepinephrine has been expanded to include the synthesis and investigation of the 2-, 5- and 6-fluoro-derivatives of the beta-adrenergic agonist, isoproterenol; the 2- and 6-fluoro-derivatives of the alpha-adrenergic antagonist, phentolamine; and the 2-, 5- and 6-fluoro-derivatives of dopamine. Preliminary results indicate that fluorine substitution on the 6 position of isoproterenol reduces the beta-adrenergic agonist potency and introduces a significant alpha-component as measured with isolated aortic strip and atrial preparations from guinea pig. The effects of 2- and 5-fluoro substitution on isoproterenol appear to be complex and will require further study.

The 2-, 5- and 6-fluoro (F) analogs of dopamine (DA) and norepinephrine (NE) were compared with the respective parent molecules for alpha-, $beta_1$- and $beta_2$-adrenergic and vascular dopamine activities in pentobarbital anesthetized dogs. 2-F and 5-F-DA are equipotent while 6-F-DA is about four-fold less active than DA in causing renal vasodilation in phenoxybenzamine-pretreated dogs (vascular DA activity). The three analogs are indistinguishable from DA for vasoconstrictor activity in the femoral vascular beds (alpha-adrenergic activity). 2-F and 6-F-DA are equipotent to DA while 5-F-DA is about two-fold more active than DA in inotropic activity ($beta_1$-adrenergic activity). In contrast, F-NE analogs show marked differential activities. 2-F-NE resembles isoproterenol in increasing cardiac contractility, lowering diastolic blood pressure and causing vasodilation in the femoral vascular bed. 5-F-NE is the most potent agent for $beta_1$-adrenergic activity and produces biphasic effects on blood pressure and the femoral vascular bed. 6-F-NE exerts no inotropic activity in doses 50- to 80-fold higher than the threshold dose of l-NE and causes vasoconstriction. Thus, fluorine substitution in either of the three positions in the benzene ring of DA induces only minor, if any, differences in receptor activation when compared with DA. On the other hand, fluorine substitution in the benzene ring of NE yields compounds with marked differential receptor activity. These data indicate that the differences between the effects of fluorine substitution on DA and NE analogs must be related to the only structural difference between the two catecholamines, the presence of a beta-hydroxyl group in NE.

The 2- and 6-fluoro-derivatives of dihydroxyphenylalanine (DOPA) and α-methyldihydroxyphenylalanine have been prepared and a study of their

metabolic and physiological properties initiated. All four amino acids are substrates for aromatic acid decarboxylase giving rise to the respective dopamine derivatives. 6-Fluoro-DOPA and 6-fluoro-alpha-methyl-DOPA lower blood pressure (tail cuff) in the spontaneously hypertensive rat. The potency of 6-fluoro-alpha-methyl-DOPA as an antihypertensive agent is similar to that obtained with alpha-methyl-DOPA.

Studies of the potential anti-leukemic histidine analog, 2-fluorohistidine (2-F-H) have continued. Initial studies on the metabolic fate of [^{3}H]2-F-H suggest the accumulation of a free metabolite in mouse tissue with an apparent half-life of 72 hours. Current evidence indicates this metabolite is 2-F-urocanic acid. In vitro studies demonstrate that the 2-F-urocanic acid is a powerful inhibitor of urocanase. A possible consequence of the formation of 2-F-urocanic acid in vivo may be a temporary interruption in the utilization of histidine as a one-carbon source for purine biosynthesis. High pressure chromatographic systems for the analysis of the metabolic products of 2-fluorohistidine have been developed and should aid in the resolution of the metabolic fate of the amino acid. Metabolic studies are in progress in the intact mouse and in isolated hepatocyte systems in vitro.

Behavioral and neurochemical characterization of the tryptophan-deficient rat have been conducted. Weanling and young (10 week) rats were placed on a variety of tryptophan deficient diets to determine the optimum diet to produce minimal brain levels of serotonin. The optimum diet yields animals with a 50 to 75% reduction of this amine in 10 days and allows maintenance of these deficient animals for an additional 20 to 30 days. The deficient animals develop a bizarre neurological syndrome coincident with the serotonin deficiency. The primary characteristic involves a distinct tremor and nearly constant activity. The normal circadian rhythm of motor activity disappears when the levels of brain serotonin reach minimal levels. The activity of the deficient animals is characterized by an increased activity in the light period and no change in the nocturnal activity. Restoration of tryptophan restores serotonin levels to normal within 1-3 hours. However, the normal circadian rhythm returns only after 3-6 days. During the intermediate period the tremor disappears and the animals go thru a period of total inactivity which persists for 1-3 days. Examination of a variety of neurochemical parameters in brain, including: stimulation of cyclic AMP accumulation by serotonin and other transmitter substances in a cerebral cortical slice preparation; uptake of ^{3}H-serotonin by brain homogenates and synaptosomal preparation; and serotonin receptor levels (high affinity building sites) in brain membrane preparations fails to reveal any significant differences between control and deficient animals. Tryptophan hydroxylase levels are reduced approximately 50% in the deficient animals. At present the specific neurochemical basis for the remarkable behavioral syndrome precipated by tryptophan deficiency remains unknown.

Role of Cyclic Nocleotides in the Nervous System

Cyclic AMP-generating systems in brain slices and in "microsac" preparations from brain are controlled in a complex manner by receptors, calcium ions, prostaglandins, guanyl nucleotides and other factors. Activation of cyclic AMP-systems in brain by α-adrenergic agonists such as 6-fluoronorepi-

nephrine occurs via an α_1-adrenergic receptor and is dependent on extracellular calcium ions. Prostaglandins of the E series affect the calcium dependency of response apparently by making extracellular calcium ions more readily available to the system. The α_1-adrenergic receptor-mediated response in cerebral cortex is virtually completely dependent on the presence of adenosine. Such data suggest the α-adrenergic receptor-mediated responses are indirect and act not directly on adenylate cyclase but indirectly by facilitating coupling and activation of adenylate cyclase by β-adrenergic receptors, A_2-adenosine receptors, and H_2-histamine receptors. However, in some brain regions, α-adrenergic receptor-mediated responses do not appear to be dependent on adenosine. It would appear the adenylate cyclase in brain tissue can be activated 1) directly by β_1- and β_2-adrenergic receptors, A_2-adenosine receptors, H_2-histamine receptors, and 2) indirectly by α_1-adrenergic receptors, H_1-histamine receptors and prostaglandin receptors in a calcium-dependent process. Certain unknown factors released from brain slices can either directly or indirectly activate adenylate cyclase. Prostaglandin receptors control the calcium-dependency of the indirect activation of adenylate cyclase. An expected inhibition of adenylate cyclase in brain slices by α_2-adrenergic receptors, A_2-adenosine receptors, prostaglandin receptors or peptide receptors could not be demonstrated.

Accumulations of cyclic AMP elicited by norepinephrine in slices of rat cerebral cortex or hypothalamus are markedly reduced after incubations with prostaglandin synthetase inhibitors such as indomethacin, aspirin, flufenamic acid, and acetoaminophen. Responses of cyclic AMP-generating systems to β-adrenergic agonists or adenosine are unchanged by indomethacin-treatment and the reduction in the norepinephrine-response appears due primarily to a loss of the α-adrenergic component. The accumulation of cyclic AMP elicited by prostaglandin E_2 (EC_{50} 4 μM) is increased twofold by indomethacin-treatment. The α-adrenergic component of the norepinephrine-response is fully restored by very low concentrations of prostaglandin E_2 (EC_{50} 20 nM). Prostaglandins of the F series have no effect on cyclic AMP generation under a variety of conditions. The α-adrenergic component of the norepinephrine-response is fully restored by high concentrations of extracellular calcium in indomethacin-treated slices. Prostaglandin E_1 allows the restoration of the α-adrenergic component by very low concentrations of calcium in indomethacin-treated slices. It appears that low levels of prostaglandins of the E series facilitate the calcium-dependent mechanism responsible for the expression of α-adrenergic receptor-mediated activation of cyclic AMP-formation in brain tissue.

2-Fluoronorepinephrine (IC_{50} 0.7 μM) is a relatively selective ligand for displacement of radioactive dihydroalprenolol from β_1-adrenergic receptors in membrane preparations from rat cerebral cortex. It is less potent (IC_{50} 10 μM) in displacing dihydroalprenolol from β_2-adrenergic receptors in rat cerebellar membranes and in displacing clonidine from α_2-adrenergic receptors in rat cerebral cortical membranes. It is much less potent (IC_{50} >100 μM) in displacing WB-4101 from α_1-adrenergic receptors in rat cerebral cortical membranes. In contrast, 6-fluoronorepinephrine is relatively selective for α-adrenergic receptors, being at least 50-200 fold more potent at such receptors than at β-adrenergic receptors. 5-Fluoronorepinephrine like norepinephrine does not exhibit remarkable selectivity towards α- and β-

adrenergic receptors. The 2-, 5- and 6- fluorodopamines are more potent ligands at α_1-adrenergic receptors than at α_2- and β-adrenergic receptors but the specificity is not markedly affected by the position of the fluorine substituent. The results suggest that the specificity exhibited by the 2- and 6-fluoronorepinephrine at adrenergic receptors is not primarily due to fluorine-induced changes in the physico-chemical properties of the aromatic ring, but instead is due to stereoselective interactions of the ethanolamine side chain of norepinephrine with fluorine at either the 2- or 6-ring position. The fluorodopamines like dopamine itself are more potent at dopaminergic than at α- or β-adrenergic receptors. The 2-, 5- and 6-fluorodopamines are all nearly equipotent with dopamine in the displacement of radioactive apomorphine from receptors associated with adenylate cyclase in striatal membranes.

Tetrahydropapaveroline, a possible metabolite of dopamine had been previously shown to have relatively high affinity for β-adrenergic receptors. These studies have been extended to α-adrenergic and dopaminergic receptors using a range of compounds to define structure activity correlations. Tetrahydropapaveroline, its optical isomers, and related compounds antagonize binding of 1) dihydroalprenolol to β_1-adrenergic receptors, 2) WB-4101 to α_1-adrenergic receptors and 3) spiroperidol to dopaminergic receptors in brain membranes. Most of the compounds are at least 10-fold more potent at the β_1-receptor than at the α_1-receptor or dopaminergic receptor. 6'-Bromo-tetrahydropapaveroline is however, very active at the α_1-receptor, quite active at the dopamine-receptor, and relatively inactive at the β_1-receptor. The levo-isomer of tetrahydropapaveroline is 100 fold more potent at the β_1-receptor than the dextro-isomer. Stereospecificity for the levo-isomer at α_1-receptor and the dopamine-receptor was only 17 fold and 5 fold, respectively. Tetrahydropapaveroline is less potent at β_2-receptors than at β_1-receptors.

Ligands for adenosine receptors have been developed. [^{3}H]Cyclohexyl-adenosine binds to adenosine receptors in brain membranes with high affinity. The receptors have low densities in such membranes and activity profiles of inhibition of binding by nucleosides and xanthines indicate the specific binding is nearly entirely to A_1-adenosine receptors. Binding is reduced by guanyl nucleotides. [^{3}H]1,3-Diethyl-8-phenylxanthine binds to the same A_1-adenosine receptors in bovine brain membranes. However, in membranes from guinea pig brain the binding of this xanthine shows lower affinity and a significant proportion of the sites have characteristics of A_2-adenosine receptors. Nucleosides have low affinities for such receptors.

Ligands for the intracellular adenosine-regulatory sites have been developed. This site, referred to as the P-site, interacts with adenosine and certain adenosine analogs to inhibit adenylate cyclase in brain membranes. As in other tissues, 2',5'-dideoxyadenosine is a potent P-site agonist in brain membrane, and adenine arabinoside a less potent agonist. [^{3}H]2',5'-Dideoxyadenosine binds with high affinity to sites in brain membranes. The sites for 2',5'-dideoxyadenosine have suprisingly high densities in brain membranes. Activity profiles of inhibition of binding by various nucleosides and the lack of inhibition by xanthines suggests that these sites are similar to P-sites, but there are certain exceptions. Thus adenosine cyclopropyl-

carboxamide and 5'-methylthioadenosine do not inhibit adenylate cyclase via the P-site, but do block binding of 2',5'dideoxyadenosine. [^{3}H]Adenine arabinoside also binds to these sites.

Calmodulin markedly activates adenylate cyclase in rat cerebral cortical and to a lesser extent in striatal membranes. Activation of adenylate cyclase, β-adrenergic, or dopaminergic receptors in such membranes appears independent of calmodulin. Both receptor and calmodulin-mediated activation of the enzyme is enhanced by guanyl nucleotides.

The interaction of calmodulin with calcium ions and with drugs has been followed by nuclear magnetic resonance spectroscopy. Transitions in conformation of this protein can be followed by spectral shifts in aromatic resonance peaks.

The Role of Benzodiazepine Receptors in the Central Nervous System

Primary cultures of fetal mouse brain and spinal cord possess benzodiazepine receptors which are pharmacologically similar to those found in the mammalian brain. These primary cultures may thus be a valid model to study the function of the benzodiazepine-GABA-chloride receptor-ionophore complex in vitro.

Mammalian retina contains both "brain-type" and "kidney (peripheral)-type" benzodiazepine receptors. These two types may be clearly resolved with the use of appropriate ligands with selectivity towards brain or peripheral-type receptor (e.g., RO-5-4864). Retina may thus prove a useful model to study the interrelationship of these two types of binding sites. Studies using intravitreal injection of kainic acid, neonatal administration of monosodium glutamate, and pink-eyed, tan-hooded rats suggest "brain-type" receptors are localized on neuronal elements of the rat retina (e.g., inner plexiform layer) and not on non-neuronal elements or photoreceptor cells.

Heterogenous populations of benzodiazepine receptors in brain were revealed using a novel alkylating agent, kenazepine. These populations of receptors have a differential chemical reactivity to this agent, which most likely resides at or near the active center of the receptor. Marked regional differences in the proportions of these receptor subtypes are observed. In vivo administration of kenazepine and another alkylating benzodiazepine (irazepine) produces a long lasting protection against pentylenetetrazole-induced convulsions when compared with equimolar administration of "classical" (non-alkylating) benzodiazepine such as diazepam.

Studies designed to explore the minimal structural requirements necessary for purines to bind to the benzodiazepine receptor were initiated. These studies were prompted by our previous finding suggesting that the purines inosine and hypoxanthine are endogenous ligands of the benzodiazepine receptor. Adenosine, a structurally related purine with a very low affinity for the receptor (greater than 2000 μM) was chosen as a starting material. Increasing the lipophilicity of adenosine by addition of halogenated heterocycles at the N-^{6} position results in an almost 100 fold increase in potency. A prototype compound (KM-12) was evaluated in this laboratory and demonstrated

to be a potent anticonvulsant. Further evaluation is being conducted by the ADD program of the NINCDS. Preliminary results appear encouraging, and this compound is now being evaluated in pre-clinical trails for long term toxicity.

Introduction of a bridged compound in the N^6-position of adenosine (e.g., bicycloheptyl or bicyclooctyl) yields compounds which increase (rather than competitively inhibit) [^{3}H]diazepam-binding in vitro. Kinetic analysis revealed this increase in [^{3}H]diazepam binding is due to an increase in site number rather than affinity. This is the first example of benzodiazepine receptor number being increased in vitro as a result of drug treatment. These increases are stereospecifically antagonized by the GABA-antagonist, bicuculline, yet are independent of GABA concentrations in the medium. These compounds do not affect [^{3}H]GABA or [^{3}H]muscimol (a GABA-agonist) binding in vitro. These data suggest that such compounds (e.g., KM-13) may increase site number in vitro by displacement of an endogenous noncompetitive inhibitor of [^{3}H]diazepam binding normally present near the benzodiazepine-GABA-chloride receptor-ionophore complex. These data further suggest that a bicuculline-sensitive site plays a permissive role in this effect, i.e., the bicuculline site must be in an "open" (unblocked) conformation for these effects to occur. In vivo administration of KM-13 to mice results in an increase in receptor number as well. This compound appears to possess long-lasting anticonvulsant, anxiolytic, and sedative properties, reminiscent of the benzodiazepines. This compound is being evaluated by the ADD program of the NINCDS.

Since certain types of essential hypertension which may be anxiety-related are treated with benzodiazepines (e.g., diazepam), the ontogeny of benzodiazepine receptors was examined in a genetically hypertensive strain of rat (SHR) and compared with a genetically matched control (W-KY). Significant differences are observed in the number of benzodiazepine receptors in both cerebral cortex and hypothalamus of SHR compared with W-KY rats. These differences may precede the development of fulminating hypertension, suggesting a causal relationship between benzodiazepine receptors and the development of certain forms of hypertension.

Further low molecular weight compound(s) have been isolated from rat brain which competitively inhibit [^{3}H]diazepam binding. These compounds are 7-20 fold more potent in inhibiting GABA-enhanced benzodiazepine binding compared with basal (non-GABA enhanced) binding. The data suggest that the GABA-benzodiazepine coupling mechanism may be an important locus for both the action of endogenous factors and pharmacologic agents.

Levels of diazepam present in the brain at a time of a profound electrophysiological effect (potentiation of adenosine-elicited depression of firing of cortical neurons) are nearly two orders of magnitude less than those needed to block adenosine uptake in vitro. These data, taken together with previous findings from our laboratory, strongly suggest that the pharmacologic effects of benzodiazepines are not related to a blockade of adenosine uptake in situ, and hence that the central effects of benzodiazepines are not dependent on activation of adenosine receptors.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31100-15 LBC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

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2.7	1.0	1.7

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) A total of over one hundred pharmacologically active alkaloids have been characterized in skin extracts of some 50 species of dendrobatid frogs. Five major classes of compounds have been defined and their pharmacology elucidated: 1) The batrachotoxins are steroid alkaloids which prevent the inactivation of voltage-dependent sodium channels in nerve and muscle; 2) The histrionicotoxins are spiropiperidine alkaloids which facilitate the conversion of chemosensitive sodium-potassium channels in nerves and effector cells to the desensitized state and block voltage-dependent potassium channels; 3) The pumiliotoxin-C class are decahydroquinoline alkaloids which block nicotinic receptors; 4) The pumiliotoxin-A class are 6-alkylidene-indolizidine alkaloids which facilitate and then block calcium-dependent excitation-secretion coupling and excitation-contraction coupling in nerve and muscle; 5) The gephyrotoxins are indolizidines and perhydrobenzoindolizidines. Certain members of this class [illegible] voltage-dependent potassium channels and muscarinic receptors. Further classes of compounds appear to be present in skin of dendrobatid frogs including deoxyhistrionicotoxins and a potent analgesic related to the pumiliotoxin-A class, but th
(cont'd)

PHS-6040
(Rev. 10-76)

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Summary of Work (Cont'd): structures and pharmacological actions remain uncertain. Other amphibians with the exception of one bufonid toad do not produce dendrobatid alkaloids. Skins of most amphibians do contain endogenous inhibitors of sodium-potassium ATPase.

Project Description:

Objectives: To elucidate the molecular basis for the pharmacological activity of batrachotoxin, histrionicotoxin, pumiliotoxins, and related substances. To isolate and elucidate the structures and molecular action of other pharmacodynamically active substances found in skin extracts of frogs and other natural sources. To explore biosynthetic pathways involved in the formation of these unique substances. To prepare radioactive ligands for the study of binding sites for these compounds. To explore possible clinical applications of dendrobatid alkaloids as myotonic and cardiotonic agents.

Methods Employed: Sensitive bioassay methods, thin layer, column, high pressure liquid, and gas chromatography have been used in the purification of the active principles, while NMR, mass spectroscopy, x-ray chrystallography, microchemical techniques, and radiochemical labeling are being utilized for structure elucidation and for the preparation of ligands for the study at binding sites. Isotopic tracer techniques have been used to study biosynthetic pathways. Biological activity has been investigated with both isolated enzyme systems, in intact cells, nerve, and muscle preparations and in intact animals.

Major Findings: Skin extracts of frogs have yielded further supplies of batrachotoxins, pumiliotoxin A, B and C, histrionicotoxins and gephyrotoxin for pharmacological investigation. Various additional alkaloids have been isolated. These include: 1) a hydroxybatrachotoxin, whose structure is under investigation, 2) three more histrionicotoxins ($C_{19}H_{25}NO$, $C_{17}H_{25}NO$, and $C_{19}H_{27}NO$), whose structures have been elucidated by nuclear magnetic resonance spectroscopy, 3) isomeric allopumiliotoxins ($C_{19}H_{33}NO_3$) which differ only in the stereochemistry of the 7-hydroxy substituent characteristic of this subclass of pumiliotoxin-A alkaloids, 4) an alkaloid ($C_{16}H_{29}NO_2$) from a bufonid toad which appears to differ from dendrobatid pumiliotoxin-A alkaloids only in the position of side chain hydroxyl group. Mass spectral analysis defines the structure but not the stereoconfiguration of the compound, 5) a tricyclic monohydroxy alkaloid ($C_{10}H_{29}NO$) which on mass spectral analysis affords a base peak ($C_7H_{13}N$) with an odd number of hydrogens, an unusual phenomenon for an alkaloid. A number of dendrobatid alkaloids of this class have now been identified, 6) an alkaloid ($C_{15}H_{25}N$) which was previously proposed to be a member of the pumiliotoxin-C class of decahydroquinolines, was shown by magnetic resonance spectroscopy to represent another class of

alkaloids, probably a deoxyhistrionicotoxin with 2-allyl and 7-vinyl substituents. A trace indolizidine alkaloid ($C_{15}H_{29}N$), present in certain dendrobatid frogs, was compared with a synthetic 2-butyl-5-propylindolizidine, synthesized by Dr. MacDonald, Vanderbilt University. Gas chromatographic mass spectral analysis indicated that the dendrobatid alkaloid has the same gross structure as the synthetic material but differs in stereochemistry. Analysis of aldehydes obtained as periodate cleavage products from pumiliotoxin-B by proton magnetic resonance spectroscopy indicate that the double bond has the major substituents trans rather than cis as previously suggested for this alkaloid. Systems for separation of dendrobatid alkaloids by high pressure liquid chromatography have been developed. Such systems should prove useful for the small scale separation and isolation of such compounds. (Edwards, Daly)

Further analysis of the analgesic compound present in trace amounts in the frog Dendrobates tricolor indicate that pyrolysis of this compound occurs during gas chromatography or mass spectrometry to yield a relatively nonpolar pyridoindolizidine with an empirical formula of $C_{10}H_{11}N_2OCl$ and that the parent analgesic alkaloid is more complex and polar in nature, perhaps related in structure to the pumiliotoxin-A class of alkaloids. (Edwards, Daly)

Alkaloids were analyzed in six further species of dendrobatid frogs, three of which represent previously undescribed species. No new classes of compounds were detected, but the data permit further evolutionary relationships within the family Dendrobatidae to be discerned. Variation of alkaloid profiles in populations of frogs and in individual frogs has, however, complicated application of such data for taxonomic purposes. It also appears that mechanisms regulating the biosynthesis of the diverse complex of dendrobatid alkaloids are controlled by many factors, both genetic and environmental. Certain species such as Phyllobates terribilis, when raised in captivity do not produce detectable levels of alkaloids. Wild individuals of this species contain high levels of batrachotoxin and are the most toxic of the dendrobatid frogs. The captive-raised frogs, although not producing detectable levels of batrachotoxin remain insensitive to its action on sodium channels in nerve and muscle. The importance of channel regulatory sites at which these alkaloids interact is suggested in that this site appears to have been only minimally altered in Phyllobates terribilis so as to prevent interaction with batrachotoxin, while remaining sensitive to other sodium conductance activators (veratridine, grayanotoxin) to which the frogs are not exposed. Offspring of the brightly colored alkaloid-producing Dendrobates tricolor and the dull colored alkaloid-free Dendrobates anthonyi produce no detectable alkaloids, nor do subsequent backcross offspring. Stress appears to increase levels of alkaloids considerably in captive Dendrobates auratus, suggesting that the role of stress in triggering alkaloid production in captive-reared dendrobatid frogs should be investigated. (Brown, Daly)

Pumiliotoxins of the A class, in particular pumiliotoxin-B, potentiate and prolong stimulus-elicited contractures of striated muscle. In frog sciatic nerve-sartorius muscle the potentiation is followed by a stimulus-dependent depression of twitch. The presence of high concentrations of

extracellular calcium prevent depression of muscle twitch. The presence of pumiliotoxin-B in nerve-muscle preparations causes single nerve stimuli to trigger repetitive endplate potentials which often summate to give an action potential and muscle twitch. Pumiliotoxin-B has no apparent effect on sodium or potassium conductances, but would appear to enhance excitation-contraction and excitation-secretion coupling by facilitating mobilization of calcium ions in nerve and muscle. (Nimitkitpaisan)

Pumiliotoxin-C and analogs inhibit neuromuscular transmission in frog sciatic nerve-sartorius muscle and rat phrenic nerve-diaphragm muscle. The most potent analog PTX-C_{II} (2,5-dipropyl-cis-decahydroquinoline) at 40 μM blocks indirectly elicited muscle twitch in 45 min in frog muscle. It has no effect on muscle membrane potential. PTX-C_{II} markedly depresses the amplitude and frequency of miniature endplate potentials and amplitude of endplate potentials as well as completely blocking extrajunctional sensitivity to iontophoretic acetylcholine in chronically denervated rat soleus muscle. The blockade of neuromuscular transmission by PTX-C_{II} is readily reversible. PTX-C_{II} is equipotent to D-tubocurarine with respect to inhibition of binding of radioactive α-bungarotoxin to acetylcholine receptors in Torpedo electroplax membranes. These decahydroquinoline alkaloids appear to represent a new class of nicotinic antagonists which prevent neuromuscular transmission by blockade of acetylcholine receptors. (Nimitkitpaisan)

Batrachotoxin, veratridine and grayanotoxin depolarize neuronal elements in a microsac preparation from guinea pig cerebral cortex. Tetrodotoxin blocks the depolarization while the polypeptide anenome toxin potentiates the response to batrachotoxin, veratridine and grayanotoxin. An additional potassium-sensitive depolarization in these microsac preparations probably represents the response of glial elements. Membrane potential was determined from uptake of radioactive triphenylmethylphosphonium ion. (McNeal, Creveling)

Batrachotoxin-A 20-α-benzoate (BTX-B) is equipotent to batrachotoxin itself in toxicity and in depolarizing nerve muscle preparations. This was unexpected since previous data had suggested the importance of a 20-α-pyrrole-3-carboxylate moiety as a prime determinant of the biological activity of batrachotoxin class of compounds. Radioactive BTX-B binds with high affinity to sites in mouse brain membranes. Specific binding represents only about ten percent of total binding at 150 nM [^{3}H]BTX-B, but the high reproducibility of a centrifugal assay permits investigation of this specific binding component which appears to have a density in brain similar to that of saxitoxin and tetrodotoxin-binding sites. Specific binding of [^{3}H]BTX-B is antagonized by compounds thought to interact with batrachotoxin-sensitive regulatory site on voltage-sensitive sodium channels with the expected order of potency: batrachotoxin > veratridine > grayanotoxin. Batrachotoxin-A also appears to displace [^{3}H]BTX-A from specific binding sites which is unexpected since this analog is not a potent activator of sodium channels. Tetrodotoxin, scorpion toxin and anenome toxin have no apparent effect on binding of [^{3}H]BTX-B. BTX-B appears to represent a new radioactive ligand for the investigation of sodium channels. (Daly)

Significance to Biomedical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, its selective action on nerve preparations, and its cardiotoxin properties gave the elucidation of its structure and correlations of structures with activity a high priority in biomedical research. Batrachotoxin has now been established as the agent of choice for the investigation of mechanisms involved in the control of sodium conductances in nerve and muscle and of the involvement of sodium-dependent processes in the control of axonal transport. Data obtained with batrachotoxin have clarified the mechanism of action of local anesthetics. Histrionicotoxin and various analogs, because of their remarkable effects on ion conductances, provide unique tools for the study of the acetylcholine-modulated ion channels in electrogenic membrane. Data with histrionicotoxins have led to the definition of a regulatory site on chemosensitive ion channels which interacts with a variety of drugs and medicinal agents including amantadine, phencyclidine and local anesthetics. Gephyrotoxin provides a new class of antagonists for potassium channels and muscarinic receptors. Antagonism of potassium channels by histrionicotoxins and gephyrotoxins provides the basis for possible development of new anti-arrhythmic agents. The pumiliotoxin-A class of alkaloids provides an important new structure for the possible development of myotonic and cardiotonic drugs. Such compounds should prove invaluable tools for the investigation of calcium-mechanisms involved in transmitter release and muscle contracture. The pumiliotoxin-C class of alkaloids provides a new structure with curare-like activity. Such compounds, because of their lipophilic character should now allow investigation of the importance of nicotinic mechanisms in the central nervous system. Other compounds from frog, fish, insect, and plant extracts also have physiological activities which warrant their structural and pharmacological investigation. Examples of such compounds occurring in frogs and toads include the samandarine, the bufodienolides, the catecholamines, indole alkylamines and histamines, and hypotensive and centrally active peptides. The potent analgesic effect of certain frog alkaloids is of particular relevance to the design of new drugs, since these alkaloids probably do not appear to interact with the central opiate receptor. An inhibitor of sodium-potassium ATPase present in skin of most frogs may prove to be an endogenous regulatory of this important enzyme.

Proposed Course of Project: The structures of further alkaloids from frogs of the general Dendrobates, Colosthetus, and Phyllobates will be elucidated with particular emphasis on compounds representing new classes of alkaloids. The pharmacological activity of batrachotoxins, pumiliotoxins, gephyrotoxins, and histrionicotoxins will be studied in further detail, including an investigation of their effects on binding of ligands to various classes of receptors. Structure activity correlations for these classes will be used to design more potent analogs. The effects of alkaloids on membrane potential and neurotransmitter release in brain microsac preparations and their interaction with local anesthetics and polypeptide toxins will be probed. Radioactive derivatives of batrachotoxin, gephyrotoxin, histrionicotoxin, and pumiliotoxin will be used to study their ultrastructural site of action. Further isolations of batrachotoxins, histrionicotoxins, pumiliotoxin and related alkaloids will be carried out to provide material for chemical

and pharmacological study. The pharmacological activity of other classes of dendrobatid alkaloids will be investigated. The nature of the inhibitors of sodium-potassium ATPase present in frog skin will be clarified. The structure of a potent analgesic alkaloid present in only trace amounts in frogs will be further delineated by the synthesis of model compounds related in structure to a pyrolysis product obtained from this alkaloid. Pharmacologically active substances will be investigated in extracts from other families of tropical frogs, from fish, insects, and plants. Further synthetic analogs of dendrobatid alkaloids will be prepared. Extracts will be examined for the presence of new types of pharmacologically active peptides and for compounds which antagonize binding of various receptor ligands.

Publications:

Daly, J.W., Tokuyama, T., Fujiwara, T., Highet, R.J., and Karle, I.L.: A new class of indolizidine alkaloids from the poison frog, Dendrobates tricolor. X-ray analysis of 8-hydroxy-8-methyl-6-(2'-methylhexylidene)-1-azabicyclo-[4.3.0]nonane. J. Am. Chem. Soc. 102: 830-836, 1980.

Myers, C.W. and Daly, J.W.: Taxonomy and ecology of Dendrobates bombetes, a new Andean poison frog with new skin toxins. Am. Mus. Novitates 2692: 1-23, 1980.

Neuwirth, M., Daly, J.W., Myers, C.W., and Tice, L.W.: Morphology of the granular secretory glands in skin of poison-dart frogs (dendrobatidae). Tissue & Cell 11: 755-771, 1979.

Flier, J., Edwards, M.W., Daly, J.W., and Myers, C.W.: Widespread occurrence in frogs and toads of skin compounds interacting with the ouabain site of Na^+, K^+-ATPase. Science 208: 503-505, 1980.

Daly, J.W., Myers, C.W., Warnick, J.E., and Albuquerque, E.X.: Levels of batrachotoxin and lack of sensitivity to its action in poison-dart frogs (Phyllobates). Science 208: 1383-1385, 1980.

Creveling, C.R., McNeal, E.T., McCulloh, D.H., and Daly, J.W.: Membrane potentials in cell-free preparations from guinea pig cerebral cortex: Effect of depolarizing agents and cyclic nucleotides. J. Neurochem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31101-12 LBC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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1.7	0.7	1.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The chemistry, biochemistry and pharmacology of biogenic amines, their amino acid precursors and transformation products has been investigated. The areas of specific interest are: 1) the immunolocalization of catechol-O-methyltransferase (COMT) in malignant and normal human and rodent tissues. 2) The effect of the substitution of fluorine on the aromatic ring of dopamine, norepinephrine, and serotonin and their amino acid precursors on the biological properties of these compounds. 3) The mechanism of toxicity and leukocytopoiesis of 2-fluorohistidine in normal and leukemic mice, and the metabolism and incorporation into protein of 2-fluorohistidine in vivo and in vitro. 4) The behaviorial and neurochemical characterization of the tryptophan-deficient rat.

PHS-6040

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Project Description:

Objectives: To study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indolealkylamines, imidazolealkylamines, and their precursors and metabolic transformation products. To develop new techniques for the investigation of membrane transport systems and receptors as related to these amines and their interaction with various drugs. To study the mechanism of action of specific neurodegenerative agents structurally related to these amines; to investigate the specific sites of the cytotoxic action of these compounds and to describe both the subcellular morphological changes and specific interaction with macromolecules which accompany this cytotoxic action. To study the histopathology of the non-neuronal effects of these cytotoxic amine derivatives. To isolate and purify enzymes operative in the biosynthesis, metabolism, and physiological function of biogenic amines. To determine the physical properties of these enzymes. To prepare specific antibodies to the development of radioimmunoassays for the measurement and localization of these proteins. To investigate the distribution of these enzymes and proteins at both the cellular and subcellular levels through the application of immunoperoxidase, ferritin, and fluorescence techniques. To determine the effects of the introduction of fluorine and other substituents on the aromatic and imidazole rings of biogenic amines on transport, metabolic, and receptor-mediated systems for biogenic amines. To investigate the metabolism, toxic actions, and antineoplastic effects of various fluorine analogs of the amino acid precursors of certain biogenic amines and to ascertain the nature and mechanism of the incorporation of these analogs into protein *in vivo* and in various cultured cell lines. To devise and test a system for the quantitative measurement of rodent behavior and to correlate such measurements with function of biogenic amine systems in brain.

Methods Employed: Drugs, biogenic amines, and various precursors have been prepared with deuterium, iodine-125, carbon-14, and tritium to study the metabolism, transport, distribution, and release of these compounds. Standard methods of synthetic organic chemistry have been used to prepare these compounds. Standard methods of protein purification and analysis have been used to purify and isolate various enzymes and proteins, as well as specific affinity labeling techniques and immunoabsorption techniques. Standard methods of histopathology employing light and electronmicroscopy have been used to examine the cytotoxic effects of neurotoxic amines and certain cell specific toxic amino acid derivatives. Standard immunodiffusion, electrophoresis and immunization techniques have been employed in the develop-

ment of various antibodies. Standard methods of cell culture have been used for the testing of various amino acid analogs on various cell lines. New techniques for the fixation of tissues to facilitate the localization of antibody-specific proteins have been developed. A new system for the quantitative analysis of rodent behavior has been explored and has required the development, through a trial and error procedure, of computer analysis to interpret the validity of the behavioral parameters.

Major Findings: Specific antibodies to rat liver catechol-O-methyltransferase (COMT) are continuing to be utilized to determine the localization of COMT at the cellular and subcellular level; to measure the rate of synthesis and degradation of the enzyme; and to measure and identify COMT in various hybrid cell lines.

An extensive study of the localization of COMT in the central nervous system of the rat is in the final stages of completion. The primary areas of localization include the oligodendroglia, astroglia and microglia; the leptomeninges, choroid plexuses, the ventricular ependyma, the tanycyte ependyma and the circumventricular organs. The major conclusions arising from this study are: 1) a non-neuronal localization of COMT; 2) oligodendrocytes and astrocytes in the densely ennervated fields of catecholaminergic terminals could provide a physiological mechanism for the restriction of transmitter migration; 3) the high concentrations of COMT in ventricular ependyma, choroid plexuses and circumventricular organs could control the accessibility of physiologically active catechols at the interfaces of brain and cerebrospinal fluid, blood and CSF, and blood and the brain parenchyma. However the metabolism of catecholamines in the immediate local of their release from axonal varicosities or terminal boutons does not appear to be a major function of COMT. Instead the localization is consistent with a) the regional restriction of the effects of catecholaminergic systems of diffuse ennervation, b) the restriction of areas in which passage of native catecholamines in which the passage between the brain parenchyma and CSF is possible, and c) separation of the effects of peripheral, circulating catecholamines from the effects of centrally derived catecholamines.

Immunocytochemical localization of COMT has been studied in the pituitary gland of rat. Intense COMT immunofluorescence is observed in pituicytes of the neural lobe, cells of the pars intermedia are negative and only the folliculo-stellate cells of the pars distalis contain COMT.

Localization studies on the adrenal gland revealed that COMT is almost exclusively in the zona glomerulosa. The adrenal medulla is devoid of COMT except in connective tissue elements.

An initial study of COMT in the eye of the adult rat reveals specific immunofluorescence of glia in the ganglion cell layer of the retina and in the choroid cells. The ciliary body is intensely fluorescent and similar in appearance to the choroid plexuses of rat brain. It is postulated that the role of COMT in this blood-aqueous barrier is similar to that of the choroid plexus at the blood-CSF barrier.

Studies have been initiated to determine the localization of COMT in the spinal cord and the peripheral nervous system. Preliminary experiments indicate the presence of COMT in Schwann cells ensheathing axons and in "glial-like" cells in the superior cervical ganglion.

The exploration of the localization of COMT in the cardiovascular system has been expanded to include both arterial and venous elements from the aorta and vena cava to capillaries. The present studies indicate that COMT is present exclusively in endothelial cells and not in smooth muscle cells. Subcellular studies utilizing the peroxidase-antiperoxidase method at present appear to confirm this localization. Because of the variance of this localization (that is the apparent absence of COMT in vascular smooth muscles), with conclusions based on biochemical and pharmacological findings this observation is being extended to include a study of COMT localization in a variety of cultured smooth muscle systems.

An initial study of the changes in the cellular localization of COMT in rat uterus during the course of pregnancy has been completed. Briefly, the enzyme is limited to epithelial cells of the uterus up to day 6. At day 7 with the implantation of the blastocyst cells containing COMT appear in the decidua in a characteristic geometric configuration on the antimesometrial side of the uterus. The appearance of COMT, as determined cytochemically, on implantation of the blastocyst, is being confirmed biochemically. The localization of COMT, estrogen hydroxylase and the tissue levels of 2-hydroxy-estradiol are being measured in a series of timed pregnancies in rat. Preliminary evidence indicates that the cytochemical changes in COMT are similar to those determined biochemically.

The immunochemical demonstration that COMT is markedly elevated in malignant breast tumors has been confirmed by direct biochemical assay of COMT, both in man and in spontaneous tumors in rat and mouse. A preliminary examination of both non-lactating rat and mouse has clearly demonstrated that the level of COMT in breast tissue increases during lactation, although the observed increase is not as dramatic as the increase seen in malignant breast tissue. The localization in ductal cells, is similar in the lactating rodent and in tumor tissue. Several rodent and human metastatic tumors of mammary origin have been examined and shown to maintain the high levels of COMT seen in the primary tumor.

Rabbit anti-rat COMT has been used to measure COMT levels by immunotitration in a comparison of the levels of COMT during the development of two genetically different rat strains. One strain Fischer-344 rats have low COMT activity which is inherited in an autosomal recessive fashion. The Fischer-344 strain was compared with the Wistar-Furth strain. The results of this study indicate that regulatory rather than temporal gene effects are responsible for the genetic differences in COMT levels seen in these two rat strains. This study has been extended to include the spontaneously hypertensive Aoki-strain rat. A comparison of the turnover rate of COMT in the liver, kidney and heart in three genetically different rat strains is in progress. Preliminary results indicate that regulatory gene effects are again responsible for the genetic differences.

Investigation of differences in the agonist specificities for the ring fluorinated derivatives of norepinephrine has been expanded to include the synthesis and investigation of the 2-, 5- and 6-fluoro-derivatives of the beta-adrenergic agonist, isoproterenol; the 2- and 6-fluoro-derivatives of the alpha-adrenergic antagonist, phentolamine; and the 2-, 5- and 6-fluoro-derivatives of dopamine. Preliminary results indicate that fluorine substitution on the 6 position of isoproterenol reduces the beta-adrenergic agonist potency and introduces a significant alpha-component as measured with isolated aortic strip and atrial preparations from guinea pig. The effects of 2- and 5-fluoro substitution on isoproterenol appear to be complex and will require further study.

The 2-, 5- and 6-fluoro (F) analogs of dopamine (DA) and norepinephrine (NE) were compared with the respective parent molecules for alpha-, $beta_1$- and $beta_2$-adrenergic and vascular dopamine activities in pentobarbital anesthetized dogs. 2-F and 5-F-DA are equipotent while 6-F-DA is about four-fold less active than DA in causing renal vasodilation in phenoxybenzamine-pretreated dogs (vascular DA activity). The three analogs are indistinguishable from DA for vasoconstrictor activity in the femoral vascular beds (alpha-adrenergic activity). 2-F and 6-F-DA are equipotent to DA while 5-F-DA is about two-fold more active than DA in inotropic activity ($beta_1$-adrenergic activity). In contrast, F-NE analogs show marked differential activities. 2-F-NE resembles isoproterenol in increasing cardiac contractility, lowering diastolic blood pressure and causing vasodilation in the femoral vascular bed. 5-F-NE is the most potent agent for $beta_1$-adrenergic activity and produces biphasic effects on blood pressure and the femoral vascular bed. 6-F-NE exerts no inotropic activity in doses 50- to 80-fold higher than the threshold dose of l-NE and causes vasoconstriction. Thus, fluorine substitution in either of the three positions in the benzene ring of DA induces only minor, if any, differences in receptor activation when compared with DA. On the other hand, fluorine substitution in the benzene ring of NE yields compounds with marked differential receptor activity. These data indicate that the differences between the effects of fluorine substitution on DA and NE analogs must be related to the only structural difference between the two catecholamines, the presence of a beta-hydroxyl group in NE.

The 2- and 6-fluoro-derivatives of dihydroxyphenylalanine (DOPA) and α-methyldihydroxyphenylalanine have been prepared and a study of their metabolic and physiological properties initiated. All four amino acids are substrates for aromatic acid decarboxylase giving rise to the respective dopamine derivatives. 6-Fluoro-DOPA and 6-fluoro-alpha-methyl-DOPA lower blood pressure (tail cuff) in the spontaneously hypertensive rat. The potency of 6-fluoro-alpha-methyl-DOPA as an antihypertensive agent is similar to that obtained with alpha-methyl-DOPA.

Studies of the potential anti-leukemic histidine analog, 2-fluorohistidine (2-F-H) have continued. Initial studies on the metabolic fate of [^{3}H]2-F-H suggest the accumulation of a free metabolite in mouse tissue with an apparent half-life of 72 hours. Current evidence indicates this metabolite

is 2-F-urocanic acid. In vitro studies demonstrate that the 2-F-urocanic acid is a powerful inhibitor of urocanase. A possible consequence of the formation of 2-F-urocanic acid in vivo may be a temporary interruption in the utilization of histidine as a one-carbon source for purine biosynthesis. High pressure chromatographic systems for the analysis of the metabolic products of 2-fluorohistidine have been developed and should aid in the resolution of the metabolic fate of the amino acid. Metabolic studies are in progress in the intact mouse and in isolated hepatocyte systems in vitro.

Behavioral and neurochemical characterization of the tryptophan-deficient rat have been conducted. Weanling and young (10 week) rats were placed on a variety of tryptophan deficient diets to determine the optimum diet to produce minimal brain levels of serotonin. The optimum diet yields animals with a 50 to 75% reduction of this amine in 10 days and allows maintenance of these deficient animals for an additional 20 to 30 days. The deficient animals develop a bizarre neurological syndrome coincident with the serotonin deficiency. The primary characteristic involves a distinct tremor and nearly constant activity. The normal circadian rhythm of motor activity disappears when the levels of brain serotonin reach minimal levels. The activity of the deficient animals is characterized by an increased activity in the light period and no change in the nocturnal activity. Restoration of tryptophan restores serotonin levels to normal within 1-3 hours. However, the normal circadian rhythm returns only after 3-6 days. During the intermediate period the tremor disappears and the animals go thru a period of total inactivity which persists for 1-3 days. Examination of a variety of neurochemical parameters in brain, including: stimulation of cyclic AMP accumulation by serotonin and other transmitter substances in a cerebral cortical slice preparation; uptake of ^{3}H-serotonin by brain homogenates and synaptosomal preparation; and serotonin receptor levels (high affinity building sites) in brain membrane preparations fails to reveal any significant differences between control and deficient animals. Tryptophan hydroxylase levels are reduced approximately 50% in the deficient animals. At present the specific neurochemical basis for the remarkable behavioral syndrome precipated by tryptophan deficiency remains unknown.

Proposed Course of the Project: The application of antibodies to COMT to studies on the localization of this enzyme will be continued emphasizing the peripheral nervous system, the pituitary, the eye and vascular smooth muscle. A major effort is in progress to determine the subcellular localization in glial cells, cells from the choroid plexus and in the proximal tubules of the kidney. The relationship of COMT in mammary cells will include a continuing study of human breast tumors with an effort to relate the type of tumor to the observed localization and level of COMT. Studies are being initiated, with one of several groups who have available isolated and classified cell types from a large repository of human breast cancer cells, to survey for COMT. A study of the level, distribution and changes in COMT which accompany the course of pregnancy in rat is in progress. The possible influence of 2-hydroxyestradiol on changes in placental COMT will be investigated. Improved techniques for the measurement and perhaps, localization of estrogen hydroxylase are being explored. When measurement

of the normal course of COMT changes during pregnancy are completed, attempts will be made to alter the period between fertilization and implantation to determine whether the invasive nature of implantation of the blastocyst triggers the rise in COMT or whether the rise in COMT thru inactivation of a catechol steroid facilitates or permits implantation.

The biological properties of fluoro-derivatives of biogenic amines and their amino acid precursors will be continued. In addition the effect of similar fluorine substitution on the specificity of antagonists of various biogenic amines will be investigated. It is hoped that the unique agonist-antagonist specificities conferred by fluorine substitution will lead to a fundamental insight into the nature of adrenergic receptors, provide valuable pharmacological tools, and possibly lead to pharmacological agents of clinical value.

The studies on 2-fluorohistidine will continue with an emphasis on determining both the nature of the metabolic fate and mechanism of toxicity of this amino acid.

Significance to Biomedical Research and the Program of the Institute: The biosynthesis, uptake, and metabolism of biogenic amines and their amino acid precursors are of fundamental importance in neurochemistry and pharmacology and have direct bearing on the function of both the central and peripheral nervous systems. The role of catechol-O-methyltransferase is important with regard to the functioning of the cardiovascular system, the detoxification of xenobiotic catechols, the inactivation of catechol steroids and in the metabolism of catecholamine transmitters in the central nervous system. An understanding of the role, localization, etc. of this enzyme may provide insight into a variety of pathological conditions in man, including essential hypertension and neoplastic disease of mammary ductal tissues. the unique agonist-specificities conferred by fluorine substitution on norepinephrine has provided a new and useful means of examining agonist-adrenergic receptor interaction and may lead to both a fundamental insight into the nature of such receptors and provide pharmacological agents of clinical value. The studies on the mechanism of the antileukemic properties of 2-fluorohistidine may provide a new therapeutic approach to the control of leukemia.

Publications:

Kirk, K.L., Cantacuzene, D., Nimitkitpaisan, Y., McCulloh, D., Padgett, W.L., Daly, J.W., and Creveling, C.R.: Synthesis and biological properties of 2-, 5-, and 6-fluoronorepinephrines. J. Med. Chem. 22: 1493-1497, 1979.

Inoue, K., Tice, L.W., and Creveling, C.R.: Immunochemical localization of catechol-O-methyltransferase of the lymphoid tissue and bone marrow of the rat. Acta Histochim. Cytochim. 12: 547, 1979.

Goldstein, D.J., Weinshilboum, R.M., Dunnette, J.H., and Creveling, C.R.: Developmental patterns of catechol-O-methyltransferase in genetically different

rat strains: Enzymatic and immunochemical studies. J. Neurochem. 34: 153-162, 1980.

Kaplan, G.P., Hartman, B.K., and Creveling, C.R.: Anti-catechol-O-methyltransferase: Demonstration of specificity, and immunological cross-reactivity with the enzyme from rat liver, kidney, brain and choroid plexuses. Neurochem. Res., in press, 1980.

Inoue, K., Tice, L.W., and Creveling, C.R.: Immunocytochemical localization of catechol-O-methyltransferase in the pregnant rat uterus. Endocrinology, in press, 1980.

Inoue, K., and Creveling, C.R.: Immunocytochemical localization of catechol-O-methyltransferase in lymphoid tissue and bone marrow of rat. Acta Histochim. Cytochim. 13: 4-14, 1980.

Goldberg, L.I., Kohli, J.D., Cantacuzene, D., Kirk, K.L., and Creveling, C.R.: Effects of ring fluorination on the cardiovascular actions of dopamine and norepinephrine in the dog. J. Pharmacol. Exp. Therap., in press, 1980.

Tice, L.W., and Creveling, C.R.: Effects of false neurotransmitters on mouse parafollicular cell function. Experientia, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 31102-09 LBC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Role of Cyclic Nucleotides in the Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The regulation of adenylate cyclase and phosphodiesterase activity by neurotransmitters, prostaglandins, adenosine, calmodulin, guanyl nucleotides, receptors P-sites, and other factors has been investigated. Such data are fundamental to an understanding of the role of cyclic nucleotides in the central nervous system. Selective ligands have been employed for the characterization of β_1- and β_2-adrenergic, α_1-adrenergic, A_1 and A_2-adenosine receptors regulating adenylate cyclase in brain tissue, and for the characterization of the inhibitory adenosine P-site associated with adenylate cyclase. Activation of cyclic AMP-systems in brain slices by α-adrenergic, H_1-histamine and serotonin receptors is dependent on calcium ions and probably involves an indirect facilitory effect on the coupling of A_2-adenosine, β-adrenergic and H_2-histamine receptors with adenylate cyclase. Prostaglandins appear to enhance availability of calcium to such systems.

PHS-6040

Project No. Z01 AM 31102-09 LBC

Objectives: To determine the factors that govern the formation, metabolism, and action of cyclic AMP and cyclic GMP in the central nervous system. To investigate the possible role of cyclic nucleotides in regulating metabolic state, calcium ion levels and translocation, and membrane properties in brain tissue. To investigate the interrelationship between the release and function of neurotransmitters with cyclic nucleotide-systems. To investigate the role of adaptive changes in cyclic nucleotide-generating systems in drug-tolerance and dependence. To investigate the role of membrane structure to the responsiveness of cyclic nucleotide-systems.

Methods Employed: Surgical techniques, environmental manipulation, drug-regimens are used to alter central function. Formation and metabolism of cyclic nucleotides in biological preparations and with isolated enzymes are studied with isotopically labeled compounds and with a variety of drugs and pharmacologically or biochemically-active agents.

Major Findings: Cyclic AMP-generating systems in brain slices and in "microsac" preparations from brain are controlled in a complex manner by receptors, calcium ions, prostaglandins, guanyl nucleotides and other factors. Activation of cyclic AMP-systems in brain by α-adrenergic agonists such as 6-fluoronorepinephrine occurs via an α_1-adrenergic receptor and is dependent on extracellular calcium ions. Prostaglandins of the E series affect the calcium dependency of response apparently by making extracellular calcium ions more readily available to the system. The α_1-adrenergic receptor-mediated response in cerebral cortex is virtually completely dependent on the presence of adenosine. Such data suggest the α-adrenergic receptor-mediated responses are indirect and act not directly on adenylate cyclase but indirectly by facilitating coupling and activation of adenylate cyclase by β-adrenergic receptors, A_2-adenosine receptors, and H_2-histamine receptors. However, in some brain regions, α-adrenergic receptor-mediated responses do not appear to be dependent on adenosine. It would appear the adenylate cyclase in brain tissue can be activated 1) directly by β_1- and β_2-adrenergic receptors, A_2-adenosine receptors, H_2-histamine receptors, and 2) indirectly by α_1-adrenergic receptors, H_1-histamine receptors and prostaglandin receptors in a calcium-dependent process. Certain unknown factors released from brain slices can either directly or indirectly activate adenylate cyclase. Prostaglandin receptors control the calcium-dependency of the indirect activation of adenylate cyclase. An expected inhibition of adenylate cyclase in brain slices by α_2-adrenergic receptors, A_2-adenosine receptors, prostaglandin receptors or peptide receptors could not be demonstrated. (Daly, Creveling, Edwards, McNeal, Partington, Padgett)

Accumulations of cyclic AMP elicited by norepinephrine in slices of rat cerebral cortex or hypothalamus are markedly reduced after incubations with prostaglandin synthetase inhibitors such as indomethacin, aspirin, flufenamic acid, and acetoaminophen. Responses of cyclic AMP-generating systems to β-adrenergic agonists or adenosine are unchanged by indomethacin-treatment and the reduction in the norepinephrine-response appears due primarily to a loss of the α-adrenergic component. The accumulation of cyclic AMP elicited

by prostaglandin E_2 (EC_{50} 4 μM) is increased twofold by indomethacin-treatment. The α-adrenergic component of the norepinephrine-response is fully restored by very low concentrations of prostaglandin E_2 (EC_{50} 20 nM). Prostaglandins of the F series have no effect on cyclic AMP generation under a variety of conditions. The α-adrenergic component of the norepinephrine-response is fully restored by high concentrations of extracellular calcium in indomethacin-treated slices. Prostaglandin E_1 allows the restoration of the α-adrenergic component by very low concentrations of calcium in indomethacin-treated slices. It appears that low levels of prostaglandins of the E series facilitate the calcium-dependent mechanism responsible for the expression of α-adrenergic receptor-mediated activation of cyclic AMP-formation in brain tissue. (Edwards, Partington)

2-Fluoronorepinephrine (IC_{50} 0.7 μM) is a relatively selective ligand for displacement of radioactive dihydroalprenolol from β_1-adrenergic receptors in membrane preparations from rat cerebral cortex. It is less potent (IC_{50} 10 μM) in displacing dihydroalprenolol from β_2-adrenergic receptors in rat cerebellar membranes and in displacing clonidine from α_2-adrenergic receptors in rat cerebral cortical membranes. It is much less potent (IC_{50} >100 μM) in displacing WB-4101 from α_1-adrenergic receptors in rat cerebral cortical membranes. In contrast, 6-fluoronorepinephrine is relatively selective for α-adrenergic receptors, being at least 50-200 fold more potent at such receptors than at β-adrenergic receptors. 5-Fluoronorepinephrine like norepinephrine does not exhibit remarkable selectivity towards α- and β-adrenergic receptors. The 2-, 5- and 6- fluorodopamines are more potent ligands at α_1-adrenergic receptors than at α_2- and β-adrenergic receptors but the specificity is not markedly affected by the position of the fluorine substituent. The results suggest that the specificity exhibited by the 2- and 6-fluoronorepinephrine at adrenergic receptors is not primarily due to fluorine-induced changes in the physico-chemical properties of the aromatic ring, but instead is due to stereoselective interactions of the ethanolamine side chain of norepinephrine with fluorine at either the 2- or 6-ring position. The fluorodopamines like dopamine itself are more potent at dopaminergic than at α- or β-adrenergic receptors. The 2-, 5- and 6-fluorodopamines are all nearly equipotent with dopamine in the displacement of radioactive apomorphine from receptors associated with adenylate cyclase in striatal membranes. (Nimitkitpaisan)

Tetrahydropapaveroline, a possible metabolite of dopamine had been previously shown to have relatively high affinity for β-adrenergic receptors. These studies have been extended to α-adrenergic and dopaminergic receptors using a range of compounds to define structure activity correlations. Tetrahydropapaveroline, its optical isomers, and related compounds antagonize binding of 1) dihydroalprenolol to β_1-adrenergic receptors, 2) WB-4101 to α_1-adrenergic receptors and 3) spiroperidol to dopaminergic receptors in brain membranes. Most of the compounds are at least 10-fold more potent at the β_1-receptor than at the α_1-receptor or dopaminergic receptor. 6'-Bromo-tetrahydropapaveroline is however, very active at the α_1-receptor, quite active at the dopamine-receptor, and relatively inactive at the β_1-receptor. The levo-isomer of tetrahydropapaveroline is 100 fold more potent at the

β_1-receptor than the dextro-isomer. Stereospecificity for the levo-isomer at α_1-receptor and the dopamine-receptor was only 17 fold and 5 fold, respectively. Tetrahydropapaveroline is less potent at β_2-receptors than at β_1-receptors. (Nimitkitpaisan)

Ligands for adenosine receptors have been developed. [^{3}H]Cyclohexyl-adenosine binds to adenosine receptors in brain membranes with high affinity. The receptors have low densities in such membranes and activity profiles of inhibition of binding by nucleosides and xanthines indicate the specific binding is nearly entirely to A_1-adenosine receptors. Binding is reduced by guanyl nucleotides. [^{3}H]1,3-Diethyl-8-phenylxanthine binds to the same A_1-adenosine receptors in bovine brain membranes. However, in membranes from guinea pig brain the binding of this xanthine shows lower affinity and a significant proportion of the sites have characteristics of A_2-adenosine receptors. Nucleosides have low affinities for such receptors. (Bruns)

Ligands for the intracellular adenosine-regulatory sites have been developed. This site, referred to as the P-site, interacts with adenosine and certain adenosine analogs to inhibit adenylate cyclase in brain membranes. As in other tissues, 2',5'-dideoxyadenosine is a potent P-site agonist in brain membrane, and adenine arabinoside a less potent agonist. [^{3}H]2',5'-Dideoxyadenosine binds with high affinity to sites in brain membranes. The sites for 2',5'-dideoxyadenosine have suprisingly high densities in brain membranes. Activity profiles of inhibition of binding by various nucleosides and the lack of inhibition by xanthines suggests that these sites are similar to P-sites, but there are certain exceptions. Thus adenosine cyclopropylcarboxamide and 5'-methylthioadenosine do not inhibit adenylate cyclase via the P-site, but do block binding of 2',5'dideoxyadenosine. [^{3}H]Adenine arabinoside also binds to these sites. (Law, Nimitkitpaisan)

Calmodulin markedly activates adenylate cyclase in rat cerebral cortical and to a lesser extent in striatal membranes. Activation of adenylate cyclase, β-adrenergic, or dopaminergic receptors in such membranes appears independent of calmodulin. Both receptor and calmodulin-mediated activation of the enzyme is enhanced by guanyl nucleotides. (Seamon)

The interaction of calmodulin with calcium ions and with drugs has been followed by nuclear magnetic resonance spectroscopy. Transitions in conformation of this protein can be followed by spectral shifts in aromatic resonance peaks. (Seamon)

Significance to Biomedical Research and the Program of the Institute: The key role of cyclases and cyclic AMP and cyclic GMP in regulating cellular activity in response to external stimuli in many biological systems make elucidation of their role in brain of fundamental importance to an understanding of function in this organ and to the development of drugs for the treatment of malfunctions involving cyclic nucleotide systems.

Proposed Course of Project: The physiological significance of receptors, calmodulin, phospholipids, gangliosides, guanyl nucleotides, and proteases

for the regulation of adenylate cyclases and phosphodiesterases will be investigated. The morphological location of the labeled pools of adenine nucleotides will be determined. The role of phosphoribosyl transferase and adenosine kinase in maintaining this pool will be explored. Secondary effects on enzymes and modulators due to elevated cyclic AMP levels in brain tissue will be investigated. The in vivo effects of drugs and the effects of lesions and hormonal or dietary alterations on the responsiveness of the cyclic AMP-systems will be further explored. The nature of the adenosine-receptor will be studied further using adenosine analogs and antagonists modified for possible affinity labeling. Other agents with selective effects on adenosine, adrenergic, serotonergic, and histaminergic systems will be sought and investigated. Further ligands for receptors associated with cyclase systems will be sought and investigated. The role of calcium ions and its interrelationship with prostaglandin and adenosine in the function of cyclic AMP-generating systems will be further investigated, both in vivo and in vitro. Correlations between cyclic GMP and cyclic AMP levels in brain slices will be attempted. Antibodies to enzymes and regulatory proteins involved in the cyclic AMP-generating systems will be prepared and effects of various regimens on the turnover, levels, and distribution of these enzymes and regulatory proteins in brain will be investigated. Further attempts to develop more selective inhibitors of cyclases, phosphodiesterases, and kinases will be made. The mechanism of amine-adenosine interactions and desensitization of receptor-adenylate cyclase complexes will be probed in slices and vesicular entities from brain. Correlations of cyclic AMP-levels, membrane potential, neurotransmitter release, and protein phosphorylation will be investigated further.

Publications:

Daly, J.W.: Adenosine and cyclic AMP-generating systems in brain tissue. In Baer, H.P. and Drummond, G.I. (Eds.): Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides. New York, Raven Press, 1979, pp. 229-241.

Partington, C.R., and Daly, J.W.: Effect of proteases and protease inhibitors on adenylate cyclase activity in rat cerebral cortical membranes. Arch. Biochem. Biophys. 198: 255-262, 1979.

Smellie, F.W., Daly, J.W., Dunwiddie, T.V., and Hoffer, B.J.: The dextro and levorotatory isomers of N-phenylisopropyladenosine: Stereospecific effects on cyclic AMP-formation and evoked synaptic responses in brain slices. Life Sci. 25: 1739-1748, 1979.

Smellie, F.W., Daly, J.W., and Wells, J.N.: 1-Isoamyl-3-isobutylxanthine: A remarkably potent agent for the potentiation of norepinephrine, histamine, and adenosine-elicited accumulation of cyclic AMP in brain slices. Life Sci. 25: 1917-1924, 1979.

Daly, J.W., Padgett, W., Nimitkitpaisan, Y., Creveling, C.R., Cantacuzene, D., and Kirk, K.L.: Fluoronorepinephrines: Specific agonists for the

activation of alpha and beta adrenergic-sensitive cyclic AMP-generating systems in brain slices. J. Pharm. Exp. Therap. 212: 382-389, 1980.

Davis, C.W., and Daly, J.W.: Activation of rat cerebral cortical 3',5'-cyclic nucleotide phosphodiesterase activity by gangliosides. Mol. Pharmacol. 17: 206-211, 1980.

Pons, F., Bruns, R.F., and Daly, J.W.: Depolarization-evoked accumulation of cyclic AMP in brain slices: The requisite intermediate adenosine is not derived from hydrolysis of released ATP. J. Neurochem. 34: 1319-1323, 1980.

Partington, C.R., Edwards, M.W., and Daly, J.W.: Calcium-dependent desensitization of adenylate cyclase in rat cerebral cortical slices. J. Neurochem. 34: 76-82, 1980.

Bruns, R.F., Pons, F., and Daly, J.W.: Glutamate- and veratridine-elicited accumulations of cyclic AMP in brain slices: A role for factors which potentiate adenosine-responsive systems. Brain Res. 189: 550-555, 1980.

Brossi, A., Rice, K.C., Mak, C.-P., Reden, J., Jacobson, A., Nimitkitpaisan, Y., Skolnick, P., and Daly, J.: Mammalian alkaloids. 8. Synthesis and biological effects of tetrahydropapaveroline related 1-benzyltetrahydroquinolines. J. Med. Chem., in press, 1980.

Daly, J.W., McNeal, E., Partington, C., Neuwirth, M., and Creveling, C.R.: Accumulations of cyclic AMP in adenine-labeled cell-free preparations from guinea pig cerebral cortex: Role of α-adrenergic and H_1-histaminergic receptors. J. Neurochem., in press, 1980.

McNeal, E.T., Creveling, C.R., and Daly, J.W.: Cyclic AMP-generating systems in cell-free preparations from guinea pig cerebral cortex: Loss of adenosine and amine responsiveness due to low levels of endogenous adenosine. J. Neurochem. in press, 1980.

Partington, C.R., Edwards, M.W., and Daly, J.W.: Regulation of cyclic AMP formation in brain tissue by α-adrenergic receptors: Requisite intermediacy of prostaglandins of the E series. Proc. Natl. Acad. Sci. USA, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31103-02 LBC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Role of Benzodiazepine Receptors in the Central Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Saturable, high affinity and stereospecific binding sites for benzodiazepines has been demonstrated both in vivo and in vitro in the mammalian central nervous system. The correlations between the binding of a series of benzodiazepines in vitro and their pharmacologic potencies as anxiolytics, anticonvulsants, and muscle relaxants strongly suggests these sites are receptors mediating the therapeutic actions of the benzodiazepines. Recent evidence suggests these receptors may be functionally linked to receptors for GABA (γ-aminobutyric acid) and a chloride-channel. This macromolecular complex may mediate physiologic functions which are analogous to the therapeutic effects of the benzodiazepines (i.e., anxiety, convulsions, muscular relaxation, sleep/wakefulness). The role and regulation of this complex are currently being investigated by: 1) isolation and identification of endogenous modulators of this complex as well as pharmacologic characterization of these modulators; 2) development of suitable in vitro models for neurochemical and electrophysiological study; 3) development of novel benzodiazepines to characterize receptor subgroups and determination of the physiologic function(s) of these subgroups; 4) development based on structural similarity to endogenous compounds of novel therapeutic agents (anxiolytics, anticonvulsants, sedatives) with benzodiazepine-

Summary of Work (Cont'd): like actions based on structural similarity to endogenous compounds.

Project Description:

Objectives: To characterize and determine the role and regulation of the benzodiazepine (receptor)-GABA (receptor)-chloride ionophore complex. To isolate, identify and evaluate endogenous substances isolated from mammalian brain which modulate this macromolecular complex. To develop suitable model systems for in vitro study of this complex. To develop novel compounds useful in studying benzodiazepine receptor function. To develop potentially useful therapeutic agents using information obtained from identification of endogenous ligands and modulators of this complex.

Methods Employed: Neurochemical characterization of the benzodiazepine-GABA-chloride receptor-ionophore complex have been studied using radioreceptor assays employing radiolabelled benzodiazepine and GABA (as well as GABA analogs). Isolation and identification of endogenous modulators of the benzodiazepine-GABA-chloride receptor-ionophore complex has been accomplished with column chromatography, high pressure liquid chromatography, and differential filtration techniques. Pharmacologic testing of compounds has largely been assessed by the anticonvulsant activity of various compounds along with measurement of spontaneous motor activity. Genetic animal models such as the spontaneous hypertensive and the pink-eyed, tan-hooded rat have been used to assess the physiologic role of this benzodiazepine-GABA-chloride receptor-ionophore complex.

Major Findings: Primary cultures of fetal mouse brain and spinal cord possess benzodiazepine receptors which are pharmacologically similar to those found in the mammalian brain. These primary cultures may thus be a valid model to study the function of the benzodiazepine-GABA-chloride receptor-ionophore complex in vitro. (Skolnick)

Mammalian retina contains both "brain-type" and "kidney (peripheral)-type" benzodiazepine receptors. These two types may be clearly resolved with the use of appropriate ligands with selectivity towards brain or peripheral-type receptor (e.g., RO-5-4864). Retina may thus prove a useful model to study the interrelationship of these two types of binding sites. Studies using intravitreal injection of kainic acid, neonatal administration of monosodium glutamate, and pink-eyed, tan-hooded rats suggest "brain-type" receptors are localized on neuronal elements of the rat retina (e.g., inner plexiform layer) and not on non-neuronal elements or photoreceptor cells. (Skolnick)

Heterogenous populations of benzodiazepine receptors in brain were revealed using a novel alkylating agent, kenazepine. These populations of receptors have a differential chemical reactivity to this agent, which most likely resides at or near the active center of the receptor. Marked regional

differences in the proportions of these receptor subtypes are observed. In vivo administration of kenazepine and another alkylating benzodiazepine (irazepine) produces a long lasting protection against pentylenetetrazole-induced convulsions when compared with equimolar administration of "classical" (non-alkylating) benzodiazepine such as diazepam. (Williams)

Studies designed to explore the minimal structural requirements necessary for purines to bind to the benzodiazepine receptor were initiated. These studies were prompted by our previous finding suggesting that the purines inosine and hypoxanthine are endogenous ligands of the benzodiazepine receptor. Adenosine, a structurally related purine with a very low affinity for the receptor (greater than 2000 μM) was chosen as a starting material. Increasing the lipophilicity of adenosine by addition of halogenated heterocycles at the N^6 position results in an almost 100 fold increase in potency. A prototype compound (KM-12) was evaluated in this laboratory and demonstrated to be a potent anticonvulsant. Further evaluation is being conducted by the ADD program of the NINCDS. Preliminary results appear encouraging, and this compound is now being evaluated in pre-clinical trails for long term toxicity. (Skolnick, Lock)

Introduction of a bridged compound in the N^6-position of adenosine (e.g., bicycloheptyl or bicyclooctyl) yields compounds which increase (rather than competitively inhibit) $[^3H]$diazepam-binding in vitro. Kinetic analysis revealed this increase in $[^3H]$diazepam binding is due to an increase in site number rather than affinity. This is the first example of benzodiazepine receptor number being increased in vitro as a result of drug treatment. These increases are stereospecifically antagonized by the GABA-antagonist, bicuculline, yet are independent of GABA concentrations in the medium. These compounds do not affect $[^3H]$GABA or $[^3H]$muscimol (a GABA-agonist) binding in vitro. These data suggest that such compounds (e.g., KM-13) may increase site number in vitro by displacement of an endogenous noncompetitive inhibitor of $[^3H]$diazepam binding normally present near the benzodiazepine-GABA-chloride receptor-ionophore complex. These data further suggest that a bicuculline-sensitive site plays a permissive role in this effect, i.e., the bicuculline site must be in an "open" (unblocked) conformation for these effects to occur. In vivo administration of KM-13 to mice results in an increase in receptor number as well. This compound appears to possess long-lasting anticonvulsant, anxiolytic, and sedative properties, reminiscent of the benzodiazepines. This compound is being evaluated by the ADD program of the NINCDS. (Skolnick, Lock)

Since certain types of essential hypertension which may be anxiety-related are treated with benzodiazepines (e.g., diazepam), the ontogeny of benzodiazepine receptors was examined in a genetically hypertensive strain of rat (SHR) and compared with a genetically matched control (W-KY). Significant differences are observed in the number of benzodiazepine receptors in both cerebral cortex and hypothalamus of SHR compared with W-KY rats. These differences may precede the development of fulminating hypertension, suggesting a causal relationship between benzodiazepine receptors and the development of certain forms of hypertension. (Tsujimura, Skolnick)

Further low molecular weight compound(s) have been isolated from rat brain which competitively inhibit [^{3}H]diazepam binding. These compounds are 7-20 fold more potent in inhibiting GABA-enhanced benzodiazepine binding compared with basal (non-GABA enhanced) binding. The data suggest that the GABA-benzodiazepine coupling mechanism may be an important locus for both the action of endogenous factors and pharmacologic agents. (Skolnick)

Levels of diazepam present in the brain at a time of a profound electrophysiological effect (potentiation of adenosine-elicited depression of firing of cortical neurons) are nearly two orders of magnitude less than those needed to block adenosine uptake in vitro. These data, taken together with previous findings from our laboratory, strongly suggest that the pharmacologic effects of benzodiazepines are not related to a blockade of adenosine uptake in situ, and hence that the central effects of benzodiazepines are not dependent on activation of adenosine receptors. (Skolnick)

Significance to Biomedical Research and the Program of the Institute: The benzodiazepines are the most widely prescribed drugs in current therapeutic use. Understanding their mechanism of action and identification of endogenous compounds which may function as "benzodiazepine-like" compounds is of fundamental importance ot the understanding of diseases such as epilepsy, anxiety-neuroses, and alcoholism, as well as development of a more fundamental understanding of brain function. In addition, these studies may provide information regarding the development of therapeutic agents superior to the benzodiazepines.

Proposed Course of Project: The significance of the benzodiazepine-GABA-chloride receptor-ionophore complex relative to the pharmacologic actions of anxiolytics other than the benzodiazepines (e.g., propanediol carbamates) and anticonvulsants other than benzodiazepines (e.g., barbiturates) will be investigated. The search for endogenous modulators of the benzodiazepine-GABA-chloride receptor-ionophore complex will be extended to include factors which may be displaced by purines such as KM-13. The role of benzodiazepine in the generation of spontaneous hypertension will be further investigated. Attempts at receptor purification will be made using the novel benzodiazepines developed in this laboratory.

Publications:

MacDonald, J.F., Barker, J.L., Paul, S.M., Marangos, P.J., and Skolnick, P.: Inosine may be an endogenous ligand for benzodiazepine receptors in cultured spinal neurons. Science 205: 715-717, 1979.

Paul, S.M., Syapin, P.J., Paugh, B.A., Moncada, V. and Skolnick, P.: CNS benzodiazepine receptors: Correlation between receptor occupation and anticonvulsant effects of diazepam. Nature 281: 688-589, 1979.

Marangos, P.J., Clark, R., Martino, A.M., Paul, S.M. and Skolnick, P.: Demonstration of two new endogenous "benzodiazepine-like" compounds from brain. Psychiatry Res. 1: 121-130, 1979.

Marangos, P.J., Paul, S.M., Goodwin, F.K., and Skolnick, P.: Putative endogenous ligands for the benzodiazepine receptor. Life Sci. 25: 1093-1102, 1979.

Paul, S.M., Marangos, P.J., Skolnick, P., and Goodwin, F.K.: Brain-specific benzodiazepine receptors and putative endogenous "benzodiazepine-like" com-compounds. Psychopharm. Bull. 16: 9-20, 1980.

Skolnick, P., Paul, S.M., and Marangos, P.J.: Brain benzodiazepine levels following intravenous administration of [^{3}H]diazepam: Relationship to the potentiation of purinergic depression of CNS neurons. Canad. J. Physiol. Pharmacol. 57: 1040-1042, 1979.

Skolnick, P., Paul, S.M., and Marangos, P.J.: Purines as endogenous ligands of the benzodiazepine receptor. Fed. Proc., in press.

Paul, S.M., Marnagos, P.J., Goodwin, F.K., and Skolnick, P.: Brain specific benzodiazepine receptors and putative endogenous "benzodiazepine-like" compounds. Biol. Psych., in press.

Huang, A., Barker, J.L., Paul, S.M., Moncada, V.Y., and Skolnick, P.: Characterization of benzodiazepine receptors in primary cultures of fetal mouse brain and spinal cord. Brain Res. 190: 485-492, 1980.

Paul, S.M., Zatz, M., and Skolnick, P.: Demonstration of "brain-specific" benzodiazepine receptors in rat retina. Brain Res. 187: 243-246, 1980.

Tallman, J., Paul, S., Skolnick, P., and Gallager, D.: Receptors for the age of anxiety: Molecular pharmacology of the benzodiazepines. Science 207: 274-281, 1980.

Williams, E.F., Rice, K.C., Paul, S.M., and Skolnick, P.: Heterogeneity of benzodiazepine receptors in the CNS demonstrated with kenazepine, an alkylating benzodiazepine. J. Neurochem., in press.

Skolnick, P., Lock, K.-L., Paul, S.M., Marangos, P.J., Jonas, R., and Irmscher, K.: Increased benzodiazepine receptor number elicited in vitro by a novel purine, EMD 28422. Eur. J. Pharmacol., in press.

Skolnick, P., Lock, L.-L., Paugh, B.A., Moncada, V., Marangos, P., Windsor, V., and Paul, S.: Pharmacologic effects of EMD 28422, a novel purine which enhances [^{3}H]diazepam binding. Pharmacol. Biochem. Behav., in press.

Paul, S.M., Marangos, P.J., and Skolnick, P.: Antianxiety agents. In Pradhan, S.N. and Maickel, R. (Eds.): Textbook of Pharmacology. New York, C.V. Mosby & Co., in press.

Paul, S.M., Marangos, P.J., and Skolnick, P.: CNS benzodiazepine receptors: Is there an endogenous ligand? In Olsen, R.W., and Yamamura, H.I. (Eds.): Psychopharmacology and Biochemistry of Neurotransmitter Receptors. New York, Elsevier North Holland, in press, 1980.

Paul, S., Marangos, P., Brownstein, M., and Skolnick, P.: Demonstration and characterization of an endogenous inhibitor of GABA-enhanced [^{3}H]diazepam binding from bovine cerebral cortex. In Costa, E., di Chiari, G., and Gessa, G.L. (Eds.): GABA and Benzodiazepine Receptors. New York, Raven Press, New York, in press, 1980.

Rehavi, M., Paul, S.M., Skolnick, P., and Goodwin, F.K.: Demonstration of specific high affinity binding sites for [^{3}H]imipramine in human brain. Life Sci., in press.

Paul, S.M., Rehavi, M., Skolnick, P., and Goodwin, F.K.: Demonstration of "high affinity" binding sites for [^{3}H]imipramine on human platelets. Life Sci., in press.

Paul, S.M., Axelrod, J., and Skolnick, P.: Estrogen-dependent efflux of catecholamines from the hypothalamus in vitro. In Usdin, F., Kopin, I., and Barchas, J. (Eds.): Catecholamines: Basic and Clinical Frontiers. New York, Pergamon Press, 1979, Vol. 2, 1206-1208.

Paul, S., Axelrod, J., and Skolnick, P.: Estrogen-dependent efflux of catecholamines from the hypothalamus in vitro. Brain Res. 178: 499-505, 1979.

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October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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7	6	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The primary goal of this project has been the elucidation of the structure of reactive metabolites which are responsible for the carcinogenic, cytotoxic, and mutagenic activity of benzo[a]pyrene and other polycyclic hydrocarbons. The approach taken consists of: i) synthesis of primary oxidative metabolites as well as selected secondary oxidative metabolites, ii) study of the metabolism of these hydrocarbons with liver microsomes, as well as with purified and reconstituted cytochrome P-450 systems with and without epoxide hydrolase, iii) tests for inherent mutagenicity of the synthetic metabolites toward bacterial and mammalian cells, iv) elucidation of the roles of the cytochrome P-450 system and epoxide hydrolase in potentiating or obliterating the mutagenicity of these metabolites, v) determination of the carcinogenic activity *in vivo* of these compounds, vi) determination of the rate and nature of the product formed when reactive metabolites such as arene oxides and diol epoxides react with biopolymers and less complex model compounds. A general theory of hydrocarbon-induced carcinogenesis has been formulated and its predictions are being tested with synthetic potential metabolites of several hydrocarbons. Based on the presently available data, the bay region theory has excellent predictive value.

Project Description:

Objectives: The objectives of the work this year have been: i) to study the basic mechanism of enzymatic oxygen activation and incorporation into organic substrates, ii) to explore enzymatic and nonenzymatic reactions of labile intermediates such as epoxides and peroxides, iii) to devise methods for the synthesis of arene oxides and epoxides, and the study of their chemical reactions, iv) to investigate the role of labile oxygenated intermediates in the mutagenicity, cytotoxicity, and carcinogenicity of xenobiotic substances, to investigate the mechanism of action of oxidative drug metabolizing enzymes and subsequent conjugating enzymes.

Methods Employed: Isotopically labeled substrates, water, and oxygen gas have been used to study the oxidation of organic compounds to labile metabolites by a variety of enzymes. Nuclear magnetic resonance spectroscopy, ultraviolet and fluorescence spectroscopy, mass spectrometry, x-ray crystallography and other physico-chemical techniques have been employed frequently in these studies. In addition to the usual techniques employed for the separation of metabolites, new high pressure liquid chromatographic techniques are being pioneered in this area. Mutagenesis is studied in bacterial and mammalian cells and correlated with whole animal carcinogenicity. Metabolism studies are conducted *in vivo*, with liver microsomal preparations, and with homogeneous preparations of epoxide hydrolase and the cytochromes P-450.

Major Findings: Previous annual reports from this Section have described a systematic approach to the study of the cytotoxicity, mutagenicity, and carcinogenicity of the ubiquitous environmental carcinogen benzo[a]pyrene (BP). Briefly these studies have consisted of i) synthesis of as many known and potential oxidative metabolites as was possible, ii) study of the metabolism of the hydrocarbon with these authentic standards in hand, iii) testing of these compounds for cytotoxic and mutagenic activity with bacterial and mammalian cells both in the presence and in the absence of added drug metabolizing systems such as cytochrome P-450 and epoxide hydrolase, and iv) evaluation of the carcinogenicity of these synthetic standards in several animal models. These studies provided evidence which indicated that (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene is the most potent carcinogenic metabolite of benzo[a]pyrene. With the first identification of an ultimate carcinogenic form for any member of the polycyclic aromatic hydrocarbon class of carcinogens, we formulated the "bay-region" theory which predicts that diol epoxides that have the epoxide group in the bay-region of the hydrocarbon will be the most chemically reactive and presumably biologically active. To test the predictions of the theory, we have continued studies on the hydrocarbons benzo[a]anthracene, chrysene, dibenzo[a,h]anthracene, and 3-methylcholanthrene, and have initiated new studies on benzo[e]pyrene, cyclopenta[c,d]pyrene, benzo[c]phenanthrene, and dibenzopyrenes.

Benzo[a]pyrene (BP). (+)-7β,8α-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro BP had been described in the previous report as the most active ultimate carcinogenic metabolite of BP known on mouse skin and in the newborn mouse

tumor model. Current studies have explored the ability of BP and 13 of its derivatives to induce fibrosarcomas after subcutaneous injection into C3H/fCum mice. This is an important tumor model since it had been used so extensively to test hydrocarbons for tumorigenic activity in the past. It is, however, an unusual model since it often fails to detect proximate and ultimate carcinogens. For example, the proximate carcinogen (+)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene had very low activity when injected in trioctanoin. This activity was increased 10- to 15-fold when the dihydrodiol was injected in DMSO or when the diacetate of the dihydrodiol was injected. The results are suggestive that unusual transport problems may be associated with the more polar oxygenated metabolites in this tumor model. Similarly, the above diol epoxide and 2-hydroxy BP, both potent carcinogens, were very weak in this tumor model. These results demonstrate the importance of evaluating the carcinogenic potential of chemicals in more than one tumor system. In the newborn mouse, 2-hydroxy BP caused about 4-fold more pulmonary tumors than BP but was equipotent to BP on skin. In none of these three tumor models has 6-hydroxy BP shown significant tumorigenic activity. This is a critical finding in light of the numerous studies which had attempted to show that this reactive molecule is an ultimate carcinogen.

In order to study the biological effects of (+)- and (-)-benzo[a]-pyrene 4,5-oxide, a synthesis of these molecules has been developed based on the resolution of (+)-cis-4,5-dihydroxy-4,5-dihydrobenzo[a]pyrene. The (-) enantiomer of benzo[a]pyrene 4,5-oxide was 1.5- to 5.5-fold more mutagenic than the (+) enantiomer in strains TA 98, TA 100, TA 1537, and TA 1538 of *Salmonella typhimurium* and in Chinese hamster V79 cells. In studies with V79 cells, the (-) enantiomer of benzo[a]pyrene 4,5-oxide was also more cytotoxic than the (+) enantiomer. When mixtures of the enantiomers were studied in V79 cells, synergistic cytotoxic and mutagenic responses were observed. The greatest cytotoxic and mutagenic effects occurred with a 3:1 mixture of the (-) and (+) enantiomers of benzo[a]pyrene 4,5-oxide, respectively.

Kinetic studies of the bay-region 7,8-diol-9,10-epoxides of BP have indicated that these molecules are quite sensitive to general acid catalyzed hydrolysis. Perhaps the most important conclusion that can be drawn from our present results is that detoxification in the cell might occur by their reactions with noncritical cellular agents by a mechanism that involves general acid catalysis, as opposed to a mechanism in which these agents act as nucleophilic scavengers. However, binding of these diol epoxides to critical cellular reagents such as DNA might also be facilitated by this same type of general acid catalysis. Thus, comparisons of second-order rate constants for the attack of nucleophiles with the solvolytic rate constants may be of limited value in predicting the biological activity of the diol epoxides. In summary, present results indicate that water is probably the cell's best protective agent against the deleterious effects of these reactive diol epoxides. Enzymic pathways such as those involving epoxide hydrolase and GSH-transferase are ineffective in the detoxication of diol epoxides.

Benzo[a]anthracene (BA) and Dibenzo[a,h]anthracene (DBA). Although DBA is known to be a more potent carcinogen than BA, the bay-region diol epoxides of BA are expected to be more reactive than those of DBA. In the previous report, we had speculated that part of the reason for this was due to the very low conversion of BA into its 3,4-dihydrodiol - the immediate metabolic precursor of the diol epoxide. Current studies of the metabolism of DBA by liver microsomes from control and induced rats as well as by a highly purified and reconstituted system have shown that its highly tumorigenic 3,4-dihydrodiol constitutes 24-28% of the total metabolism of the hydrocarbon. This is the most extensive known conversion of a hydrocarbon into a proximate dihydrodiol carcinogen. Further metabolism of the 3,4-dihydrodiol occurred to form 3,4-diol-1,2-epoxides which were detected as their tetraol solvolysis products. These same products could be identified as metabolites when DBA was used as the substrate. Thus, DBA undergoes rather facile conversion into its bay-region diol epoxides. This facile metabolism of DBA combined with the low conversion of BA into its 3,4-dihydrodiol is thought to be a major determinant in the relative activity of the two hydrocarbons.

Through a combination of chemical degradation and spectral techniques, it has not been possible to assign absolute configurations to the 3,4-, 5,6-, 8,9-, and 10,11-dihydrodiols of BA. The predominant enantiomers of the metabolically formed dihydrodiols have [R,R] absolute configuration. Planned metabolism studies of the optically active benzo-ring dihydrodiols will seek to determine if the cytochromes P-450 selectively produce diol epoxide diastereomers in which the benzylic hydroxyl group is trans to the epoxide oxygen (diol epoxide-2 series), as was the case for the BP 7,8-dihydrodiol. Since studies in the newborn mouse have shown that the (-)-BA 3,4-dihydrodiol and the BA 3,4-diol-1,2-epoxide-2 are the most tumorigenic stereoisomers, it seems probable that the most active diol epoxide of BA will directly superimpose on that from BP. Thus of four possible stereoisomers, the most tumorigenic isomer is that which is metabolically predominant.

Benzo[e]pyrene (B[e]P). Tumor studies both on mouse skin and in newborn mice have shown that B[e]P as well as its 4,5- and 9,10-dihydrodiols are practically inactive as carcinogens. Several approaches were undertaken in order to explain the lack of activity of the parent hydrocarbon. The first of these was a renewed attempt to prepare the diastereomeric bay-region 9,10-diol-11,12-epoxides. Earlier studies had shown that mixtures were formed which could not be separated without concomitant decomposition. A major advance was made with the finding that high performance liquid chromatography could be used to separate the mixture. Bay-region diol epoxides of several hydrocarbons were found to chromatograph intact on Du Pont SIL columns eluted with mixtures of tetrahydrofuran and hexane. Once the B[e]P diol epoxides were obtained in pure form, nmr spectroscopy established that both diastereomers exist predominantly in the conformation with quasi-diaxial hydroxyl groups. This is the first example of a diol epoxide-2 diastereomer in which the hydroxyl groups are quasi-diaxial. Both isomers were found to be very weak mutagens toward bacterial and mammalian cells - presumably because of the quasi-diaxial hydroxyl groups since the 9,10-

epoxide of 9,10,11,12-tetrahydro B[e]P is highly mutagenic. Metabolic factors also contribute to the lack of tumorigenicity of B[e]P. The possible proximate carcinogenic metabolite, B[e]P 9,10-dihydrodiol, was formed by liver microsomes but constituted less than 1% of the total metabolites. Furthermore, no more than trace amounts of the bay-region diol epoxides were formed from this dihydrodiol, presumably because it has quasi-diaxial hydroxyl groups.

Phenanthrene and Chrysene. Studies on the racemic dihydrodiols and bay-region diol epoxides of chrysene and phenanthrene are now essentially complete. The bay-region 1,2-diol-3,4-epoxides of chrysene were as much as 40-fold more mutagenic toward mammalian and bacterial cells than was chrysene K-region 5,6-oxide. The phenanthrene diol epoxides were much less mutagenic than the corresponding chrysene diol epoxides. In initiation-promotion experiments on mouse skin, phenanthrene and its three metabolically possible trans dihydrodiols were found to be practically inactive as tumorigenic agents. In the newborn mouse tumor model, (±)-1β, 2α-dihydroxy-3α,4α-epoxy-1,2,3,4-tetrahydrochrysene (diol epoxide-2) was the most potent chrysene derivative tested. It induced pulmonary tumors in 98% of the mice, with an average of 15.9 tumors/mouse. (±)-1β,2α-Dihydroxy-3β,4β-epoxy-1,2,3,4-tetrahydrochrysene (diol epoxide-1), had little if any tumorigenic activity at the dose tested. trans-1,2-Dihydroxy-1,2-dihydrochrysene and 1,2-dihydrochrysene, the immediate metabolic precursors of a bay-region diol-epoxide and a bay-region tetrahydroepoxide, respectively, were the next most active chrysene derivatives tested. Phenanthrene and its derivatives were less tumorigenic than chrysene and its derivatives. Phenanthrene, 1,2-dihydrophenanthrene, and the two diastereomeric bay-region diol epoxides of phenanthrene had little or no tumorigenic activity, but the bay-region tetrahydroepoxide, 3,4-epoxy-1,2,3,4-tetrahydrophenanthrene, had some activity.

3-Methylcholanthrene (MC). In the previous report, we had described the isolation of an MC 9,10-dihydrodiol which was also hydroxylated on the C-1 methylene group. This dihydrodiol with a bay-region double bond was readily activated by liver microsomal enzymes to potent mutagens. In initiation-promotion experiments on mouse skin, comparisons of the percentage of mice with tumors and the average number of tumors per mouse indicated that 3-methylcholanthrene, 2-hydroxy-3-methylcholanthrene, 3-methylcholanthrene-2-one, and a 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene diastereomer were approximately equipotent as tumor initiators. In the newborn mouse tumor model, 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene was the most tumorigenic compound tested. 3-Methylcholanthrene and 3-methylcholanthrene-2-one were the next most active compounds, and they had approximately one-half to one-third of the activity of 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene when the data were expressed as pulmonary tumors per mouse. 1,9,10-Trihydroxy-9,10-dihydro-3-methylcholanthrene also produced hepatic tumors. 3-Methylcholanthrene and 2-hydroxy-3-methycholanthrene had one-fourth to one-tenth the activity of 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene in the liver. The other 3-methylcholanthrene metabolites were essentially inactive in producing hepatic tumors. The high tumorigenic

activity of 1,9,10-trihydroxy-9,10-dihydro-3-methylcholanthrene on mouse skin and in newborn mice provides evidence for bay-region activation of 3-methylcholanthrene to an ultimate carcinogen. The data also indicate that other metabolites of 3-methylcholanthrene may play a role in the carcinogenicity of this polycyclic aromatic hydrocarbon.

Cyclopentapyrene (CPP). Substantial interest has developed in the hydrocarbon CPP because of its wide distribution in the environment and the ease with which it is metabolically activated to mutagens. Only one tumor study had been done which suggested that the hydrocarbon might be tumorigenic. Since CPP has no bay-region, some authors claimed it was an exception to the bay-region theory. When an appropriate set of compounds were tested, it was established that the metabolism-induced mutagenicity of CPP was in no way unique or unusually high. Benzo[a]pyrene was found to be at least 20-fold more active as a tumor-initiator on mouse skin than was CPP, which had activity comparable to BA. CPP is significantly less active than chrysene. Thus, contrary to inferences made from its high mutagenic activity, CPP is a very weak tumor-initiator on mouse skin. The substantial effort which NCI is putting into this compound may not be justified.

Dihydrodiol Synthesis. In the past year, the Section has entered into the study of three new hydrocarbons to test the bay-region theory. Two of these are the potent carcinogens dibenzo[a,h] and [a,i]pyrene. Fairly direct methods have been utilized to synthesize the key dihydrodiols with bay-region double bonds for each of these, and the dihydrodiols have been further transformed into their bay-region diol epoxides in the isomer-2 series. Thus far, several methods have failed to yield their isomer-1 congeners. The other hydrocarbon studied was the very weak carcinogen benzo[c]phenanthrene. The study of this compound was undertaken because it has a unique, highly hindered bay-region between C-1 and C-12. Because of the high degree of steric hindrance in this area, it was impossible to synthesize its bay-region 1,2-dihydrodiol by direct methods. A direct autoxidation route did, however, prove successful. The 3,4- and 5,6-dihydrodiols were obtained by direct methods.

Epoxide Hydrolase. Human liver microsomal expoxide hydrolase has been highly purified to a specific activity (570 to 620 nmol/min/mg of protein) comparable to that of the rat enzyme using styrene oxide as substrate. Like the purified rat liver microsomal epoxide hydrolase, the human enzyme has a minimum molecular weight of 49,000 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and exhibits broad substrate specificity toward a variety of alkene and arene oxides. Despite these similarities, the human and rat enzymes are different proteins as judged by their immunochemical properties as well as their relative catalytic activities toward certain substrates.

Highly purified rat hepatic microsomal epoxide hydrolase, which had been purified in the presence of proteolytic enzyme inhibitors, was subjected to carboxypeptidase Y digestion, automated Edman degradation, and carbohydrate analysis. Carboxypeptidase Y digestion resulted in the near stoichiometric release of leucine, the COOH-terminal amino acid. Automated Edman degradation

permitted the identification of the first 20 amino acid residues of epoxide hydrolase. Methionine was identified as the NH_2-terminal residue. The NH_2-terminal region of epoxide hydrolase is similar in hydrophobicity to the NH_2-terminal precursor segments of several secretory proteins and the NH_2-terminal regions of several microsomal cytochromes P-450. Carbohydrate analyses of the enzyme revealed the presence of 0.5 to 1.0 mol of mannose/ 50,000 g of protein. These results provide evidence for the presence of a single polypeptide chain in our purified enzyme preparations and suggest that there may be only one enzymic form of epoxide hydrolase in microsomes from phenobarbital-treated rats.

We have developed a rapid, continuous, and highly sensitive fluorescence assay for the measurement of epoxide hydrolase activity. The method is based on the large differences between the fluorescence spectra of certain K-region arene oxides and their corresponding trans-dihydrodiols. Enzymatic hydration of K-region arene oxides of phenanthrene, pyrene, benzo[a]pyrene, and 7,12-dimethylbenzo[a]anthracene was studied. The assay was most sensitive with benzo[a]pyrene-4,5-oxide as substrate. With 10 μM benzo[a]pyrene-4,5-oxide, enzymatic rates of 30 pmol of dihydrodiol/min/mg of protein are three to five times those of the blank without enzyme. The fluorometric method described has been used to study site-directed inhibitors of epoxide hydrolase and the stereoselective hydration of racemic arene oxides.

Significance to Biomedical Research and the Program of the Institute: Although enzymatic oxidation and drug metabolism have long been held to constitute a means of detoxication, results of the type described here make it quite clear that certain of these pathways for drugs and environmental chemicals result in toxic and carcinogenic metabolites. Once the nature of these active metabolites has been identified, it may be possible to predict which industrial and agricultural chemicals are unsafe for use. The potential impact on the design of safe drugs is clear. A workable theory of hydrocarbon-induced carcinogenicity with predictive properties is of immense practical value.

Proposed Course of Project: A primary area of interest in the coming year will be the detailed exploration of the role of absolute stereochemistry in the metabolism and carcinogenicity of the polycyclic aromatic hydrocarbons. To this end, the absolute stereochemistry of the dihydrodiols form phenanthrene and chrysene will be determined. Enantiomerically pure bay-region diol epoxides will be prepared from BA and chrysene in order to determine their relative biological activity. Tumorigenicity of the bay-region diol epoxides of the dibenzopyrenes, B[e]P and benzo[c]phenanthrene will be tested. Studies will continue on the mechanism of action of epoxide hydrolase, and elucidation of the metabolism of variously fluorinated hydrocarbons will be undertaken.

Publications:

Chang, R.L., Wislocki, P.G., Kapitulnik, J., Wood, A.W., Levin, W.: Yagi, H., Mah, H.D., Jerina, D.M., and Conney, A.H.: Carcinogenicity of 2-hydroxybenzo[a]pyrene and 6-hydroxybenzo[a]pyrene in newborn mice. Cancer Res. 39: 2660-2664, 1979.

Wood, A.W., Levin, W., Thakker, D.R., Yagi, H., Chang, R.L., Ryan, D.E., Thomas, P.E., Dansette, P.M., Whittaker, N., Turujman, S., Lehr, R.E., Kumar, S., Jerina, D.M., and Conney, A.H.: Biological activity of benzo[e]pyrene: An assessment based on mutagenic activities and metabolic profiles of the polycyclic hydrocarbon and its derivatives. J. Biol. Chem. 254: 4408-4415, 1979.

Akhtar, M.N., Hamilton, J.G., Boyd, D.R., Braunstein, A., Seifried, H. E., and Jerina, D.M.: Anthracene 1,2-oxide: Synthesis and role in the metabolism of anthracene by mammals. J. Chem. Soc. Perkin I: 1442-1446, 1979.

Lu, A.Y.H., Thomas, P.E., Ryan, D., Jerina, D.M., and Levin, W.: Purification of human liver microsomal epoxide hydrase: Differences in the properties of the human and rat enzymes. J. Biol. Chem. 254: 5878-5881, 1979.

Wislocki, P.G., Buening, M.K., Levin, W., Lehr, R.E., Thakker, D.R., Jerina, D.M., and Conney, A.H.: Tumorigenicity of the diastereomeric benz[a]anthracene 3,4-diol-1,2-epoxides and the (+)- and (-)-enantiomers of benz[a]anthracene 3,4-dihydrodiol in newborn mice. J. Nat. Cancer Inst. 63: 201-204, 1979.

Chang, R.L., Wood, A.W., Levin, W., Mah, H.D., Thakker, D.R., Jerina, D. M., and Conney, A.H.: Differences in mutagenicity and cytotoxicity of (+) and (-)-benzo[a]pyrene 4,5-oxide: A synergistic interaction of enantiomers. Proc. Natl. Acad. Sci. USA 76: 4280-4284, 1979.

Dansette, P.M., DuBois, G.C., and Jerina, D.M.: Continuous fluorometric assay of epoxide hydrase activity. Anal. Biochem. 97: 340-345, 1979.

Lehr, R.E., Taylor, C.W., Kumar, S., Levin, W., Chang, R., Wood, A.W., Conney, A.H., Thakker, D.R., Yagi, H., Mah, H.D., and Jerina, D.M.: Differences in metabolism provide a basis for the low mutagenicity and carcinogenicity of benzo[e]pyrene compared to benzo[a]pyrene. In Jones, P. W. and Leber, P. (Eds.): Polynuclear Aromatic Hydrocarbons: Third International Symposium on Chemistry and Biology, Carcinogenesis and Mutagenesis. Ann Arbor, Michigan, Ann Arbor Science Publishers, Inc.,1979, Vol. 4, pp. 3749.

Thakker, D.R., Nordqvist, M., Yagi, H., Levin, W., Ryan, D., Thomas, P., Conney, A.H., and Jerina, D.M.: Comparative metabolism of a series of polycyclic aromatic hydrocarbons by rat liver microsomes and purified cytochrome P-450. In Jones, P.W. and Leber, P. (Eds.): Polynuclear Aromatic Hydrocarbons: Third International Symposium on Chemistry and Biology, Carcinogenesis and Mutagenesis. Ann Arbor, Michigan, Ann Arbor Science Publishers, Inc., 1979, Vol. 4, pp. 455-472.

Wood, A.W., Levin, W., Chang, R.L., Yagi, H., Thakker, D.R., Lehr, R.E., Jerina, D.M., and Conney, A.H.: Bay region activation of carcinogenic polycyclic hydrocarbons. In Jones, P.W. and Leber, P. (Eds.): Polynuclear Aromatic Hydrocarbons: Third International Symposium on Chemistry and Biology, Carcinogenesis and Mutagenesis. Ann Arbor, Michigan, Ann Arbor Science Publishers, Inc. 1979, Vol. 4, pp. 531551.

Levin, W., Buening, M.K., Wood, A.W., Chang, R.L., Thakker, D.R., Jerina, D.M., and Conney, A.H.: Tumorigenic activity of 3-methylchol-anthrene metabolites on mouse skin and in newborn mice. Cancer Res. 39: 3549-3553, 1979.

Nordqvist, M., Thakker, D.R., Levin, W., Yagi, H., Ryan, D.E., Thomas, P. E., Conney, A.H., and Jerina, D.M.: The highly tumorigenic 3,4 dihydrodiol is a principal metabolite formed from dibenzo[a,h]anthracene by liver enzymes. Mol. Pharmacol. 16: 634-655, 1979.

Yagi, H., Thakker, D.R., Lehr, R.E., and Jerina, D.M.: Benzo-ring diol epoxides of benzo[e]pyrene and triphenylene. J. Org. Chem. 44: 3439-3442, 1979.

Thakker, D.R., Levin, W., Yagi, H., Turujman, S., Kapadia, D., Conney, A. H., and Jerina, D.M.: Absolute stereochemistry of the trans dihydrodiols formed from benzo[a]anthracene by liver microsomes. Chem.-Biol. Interact. 27: 145-161, 1979.

Wood, A.W., Chang, R.L., Levin, W., Ryan, D.E., Thomas, P.E., Mah, H. D., Karle, J.M., Yagi, H., Jerina, D.M., and Conney, A.H.: Mutagenicity and tumorigenicity of phenanthrene and chrysene epoxides and diol epoxides. Cancer Res. 39: 4069-4077, 1979.

Croisy-Delcey, M., Ittah, Y., and Jerina, D.M.: Synthesis of benzo[c]-phenanthrene dihydrodiols. Tetrahedron Lett.: 2849-2852, 1979.

DuBois, G.C., Appella, E., Armstrong, R., Levin, W., Lu, A.Y.H., and Jerina, D.M.: Hepatic microsomal epoxide hydrase: Chemical evidence for a single polypeptide chain. J. Biol. Chem. 254: 6240-6243, 1979.

Nordqvist, M., Thakker, D.R., Yagi, H., Lehr, R.E., Wood, A.W., Levin, W., Conney, A.H., and Jerina, D.M.: Evidence in support of the bay region theory as a basis for the carcinogenic activity of polycyclic aromatic hydrocarbons. In Bhatnagar, R.S. (Ed.): Molecular Basis of Environmental Toxicity. Ann Arbor, Michigan, Ann Arbor Science Publishers, Inc., 1979, pp. 329-357.

Whalen, D.L., Ross, A.M., Montemarano, J.A., Thakker, D.R., Yagi, H., and Jerina, D.M.: General acid catalysis in the hydrolysis of benzo[a]pyrene 7,8-diol-9,10-epoxides. J. Am. Chem. Soc. 101: 5086-5088, 1979.

Lehr, R.E., Kumar, S., Cohenour, P.T., and Jerina, D.M.: Dihydrodiols and diol epoxides of dibenzo[a,i]- and [a,h] pyrene. Tetrahedron Lett.: 3819-3821, 1979.

Buening, M.K., Levin, W., Karle, J.M., Yagi, H., Jerina, D.M., and Conney, A.H.: Tumorigenicity of bay-region epoxides and other derivatives of chrysene and phenanthrene in newborn mice. Cancer Res. 39: 5063-5068, 1979.

Buening, M.K., Levin, W., Wood, A.W., Chang, R.L., Lehr, R.E., Taylor, C.W., Yagi, H., Jerina, D.M., and Conney, A.H.: Tumorigenic activity of benzo[e]-pyrene derivatives on mouse skin and in newborn mice. Cancer Res. 40: 203-206, 1980.

Wood, A.W., Levin, W., Chang, R.L., Huang, M.T., Ryan, D.E., Thomas, P. E., Lehr, R.E., Kumar, S., Koreeda, M., Akagi, H., Ittah, Y., Dansette, P., Yagi, H., Jerina, D.M. and Conney, A.H.: Mutagenicity and tumor initiating activity of cyclopenta[c,d]pyrene and structurally related compounds. Cancer Res. 40: 642-649, 1980.

Kouri, R.E., Wood, A.W., Levin, W., Rude, T.H., Yagi, H., Mah, H.D., Jerina, D.M., and Conney, A.H.: Subcutaneous carcinogenicity of benzo[a]pyrene and thirteen benzo[a]pyrene derivatives in C3H/fCUM mice. J. Nat. Cancer Inst. 64: 617-623, 1980.

Thakker, D.R., Yagi, H., Levin, W., Wood, A.W., Conney, A.H., and Jerina, D.M.: Metabolic activation of polycyclic aromatic hydrocarbons at the bay-region. In Breimer, D.D. (Ed.): Towards Better Safety of Drugs and Pharmaceutical Products. Amsterdam, Elsevier/North-Holland Biomedical Press, pp. 207-226, 1980.

Wood, A.W., Chang, R.L., Huang, M.T., Levin, W., Lehr, R.E., Kumar, S., Thakker, D.R., Yagi, H., Jerina, D.M., and Conney, A.H.: Mutagenicity of benzo[e]pyrene and triphenylene tetrahydroepoxides and diol epoxides in bacterial and mammalian cells. Cancer Res. 40: 1985-1989, 1980.

MacLeod, M.C., Levin, W., Conney, A.H., Lehr, R.E., Mansfield, B.K., Jerina, D.M., and Selkirk, J.K.: Metabolism of benzo[e]pyrene by rat liver microsomal enzymes. Carcinogenesis 1: 165-173, 1980.

Annual Report of the

Mathematical Research Branch

National Institute of Arthritis, Metabolism, and Digestive Diseases

A vector norm ϕ is said to be absolute if it depends only on the absolute values of the vector components and monotonic if $|x_i| \geq |y_i|$ implies $\phi(x) \geq \phi(y)$ where x_i and y_i are the components of the vectors x and y. It is well known that these properties are equivalent and in turn equivalent to the property that the norm $||A||$ induced by ϕ is axis-oriented, i.e. $||D|| = \max |d_{ii}|$ for every diagonal matrix D. We have extended this classical theorem as follows: Let N be any generalized matrix norm (not necessarily an induced norm). Then if N is axis-oriented any vector norm consistent with N is an absolute (hence monotonic) vector norm. (John Z. Hearon).

On the basis of results reported last year we have shown the following: Given a compartmental system let $\bar{t}_i$ be the mean residence time for particles initially in the i^{th} compartment. If v_i is the arbitrary constant input to the i^{th} compartment, then in the steady state the mean residence time under inputs v_i is $\tau = \Sigma v_i \bar{t}_i / \Sigma v_i$. Further, if S is the steady-state amount in the system and $R = \Sigma v_i$ the total input rate then $S = R\tau$. This theorem relating mean life, stationary content and input has been derived with no restrictions whatever upon the distribution of inputs and exits (cites of excretion). (John Z. Hearon).

A caricature of a system with a periodic state space is proposed of the form: $u_t = f(u, \lambda)$ where u is the state variable, f is periodic in u, and λ is an adjustable parameter. Such "devices" occur in many biological systems such as nerve axons, ovulation, etc. For λ small $f(u, \lambda) > 0$ and the system is like an oscillator. For λ large enough $f(u, \lambda)$ has a pair of roots and behaves as an excitable system. When many of these devices are coupled together on a ring of arbitrary length, it is possible to observe waves. Existence theorems are proven which show "pulse waves" and "periodic waves" when the system is excitable and periodic waves or oscillations, when $f(u, \lambda) > 0$. A paper has been recently submitted to J. Math. Biol. (Ermentrout and Rinzel).

The numerical stability of a class of solutions to the equation:

$$z_t = z_{xx} + z(a + bz\bar{z})$$

has been determined. In particular, certain solutions of the form

$$r(x) \exp(i(\bar{\sigma}t + a(x)))$$

are shown to be numerically unstable and therefore their relevance to biological behavior is in question. A short note has been submitted to J. Math. Biol. (G. B. Ermentrout and J. Rinzel).

The question of stability of small amplitude periodic wavetrains in a

reacting-diffusing chemical system has been resolved. A two-parameter family of waves, parametrized by the bifurcation parameter, γ, and the wave number, k is described. Restictions on k and γ are given within which the resultant waves are stable. Longwaves are constructed for these systems near a Hopf bifurcation and their stability is determined. A short version of this has appeared in the Proceedings of the Conference on Nonlinear POE's. The extended version has been submitted to Quart. Appl. Math. (G. B. Ermentrout).

The stability and bifurcation results of earlier work have been generalized and extended to arbitrary one-dimensional homogeneous, stationary, isotropic media. There are five distinct cases which lead respectively to unstable fast wavefronts, stable slow wavefronts, stationary patterns, longwaves and oscillations, and short traveling or standing wavetrains. The parameters of the system are used to select between these five modes and their stability is also determined. The results have been submitted to Physica D. (G. B. Ermentrout).

Work on epithelial transport has focused on the mathematical formulation of solute-linked water transport. An abstract analysis, applicable to a broad class of epithelial models, has been developed that assigns to any of these models an osmotic coupling coefficient. It is shown that coupling must be tight in order for a model to simulate an epithelium such as rabbit gall bladder, which can transport water both isotonically and against a substantial osmotic gradient. Previously, the compartment model of the lateral intercellular space was found to provide an adequate simulation of osmotic water transport. A "standing-gradient" type model of the interspace has now been analyzed according to our general framework. Such models have been published to indicate a possible mechanism for isotonic transport. We find that although the coupling may be tight for a standing gradient system, this model appears unable to realistically simulate water transport against an adverse osmotic gradient. For both the compartment and standing-gradient models, it is shown that tight coupling renders inescapable the conclusion that whole epithelial water permeability (determined experimentally) is a gross underestimate of cell membrane water permeability. This material has been prepared for publication as two papers and, in collaboration with Dr. K. R. Spring (LKEM), a chapter for a volume on membrane transport. It has been presented in seminars to physiologists and at the Gordon Research Conference on Theoretical Biology (1980). (Dr. A. Weinstein and Dr. J. L. Stephenson).

The existence of multiple steady states for certain models of the renal medulla has been recognized by us for some time. Analysis of the equations describing central core models has shown this (J. Stephenson, R. Mejia and B. Kellogg, Multiple Solutions in Models of the Renal Medulla, FASEB Abs #560, Vol. 39, Number 6, May 1, 1980). Numerical techniques have been developed to (i) obtain all solutions to a model for a given set of parameters and (ii) to follow the solution surface as a function of any one of the model's parameters (R. Mejia and J. L. Stephenson, Multiple Solutions of Convection - Diffusion Equations Describing the Renal Concentrating Mechanism, submitted for presentation). Whether these multiple solutions are modelling artifacts or have some physiological significance remains to be established. Three criteria have thus far been identified for this purpose: (i) the time stability of solutions, (ii) their thermodynamic energy requirements, and (iii) the shape of the solution surface and the ability of physiologic systems to proceed from one state to another on this surface.

An adaptive numerical scheme for solving the nonlinear differential equations which describe urine formation in the whole kidney was presented at the 1979 SIAM Fall Meeting (R. Mejia and J. L. Stephenson, An Alternating Method for Solution of a Multipoint Boundary Value Problem). This is an extension to the algorithm used to solve the time dependent kidney equation for many nephrons (R. Mejia and J. L. Stephenson, Numerical Solutions of Multinephron Kidney Equations, J. Comput. Phys. Vol. 32, Number 2, pp. 235-246, 1979).

A six tube vasa recta model has been solved using a number of numerical schemes (R. Mejia, J. L. Stephenson and R. J. LeVeque, A Test Problem for Kidney Models, Math. Biosci., to appear) and published as a tool for investigators to use in validating their numerics when solving similar convection-diffusion problems. (R. Mejia and J. L. Stephenson).

In collaboration with Dr. E. W. Stephenson, NIAMDD, the diffusion of calcium into skinned muscle fibers has been analyzed. This diffusion is coupled with two reactions 1) A rapid initially irreversible uptake by the sarcoplasmic reticulum, and 2) a reversible binding reaction to the muscle protein troponin. Both of these reactions reduce the rate at which calcium diffuses into the fiber interior and so force generation. Some of the results of this preliminary study were incorporated into a review for the AJP:Cell Physiology by Dr. E. W. Stephenson on the skinned fiber preparation (J. L. Stephenson collaborating with E. W. Stephenson, NIAMDD).

In general, nerve impulse propagation speed and interspike interval (ISI) change during propagation. This qualitative phenomenon may be inferred from the dispersion relation (speed versus ISI) for steadily propagating periodic wave train solutions of nerve conduction equations. Recently we have quantitatively applied this relation in an approximate kinematic description for the changing speeds and ISI's of individual pulses. For the Hodgkin-Huxley (HH) model, several numerical comparisons reveal excellent agreement between easily computed, kinematic pulse trajectories and trajectories obtained from numerical solutions of the full HH cable equations. These and previous dispersion calculations, including observations of supernormal speeds, for the HH model have been described and related to experiments (R. Miller and J. Rinzel, submitted for publication). For some models, e.g. a FitzHugh-Nagumo model in some parameter ranges, the dispersion relation is strikingly non-monotone with a range of ISI's where speed may exceed the solitary pulse speed by 50%. In such a case, kinematic calculations illustrate how a train of uniformly timed stimuli may generate pulses which, during propagation, develop into locked paris with alternating short and long ISI's. In contrast, if the dispersion relation is primarily monotone (speed increasing with ISI) then ISI's typically tend toward uniformity during propagation. Numerically computed examples illustrate these qualitative differences between two classes of models and provide further empirical support for the kinematic approximation. A paper "Impulse propagation in excitable systems" was presented at the Mathematics Research Center Advanced Seminar. (John Rinzel).

For some parameter ranges, the FitzHugh-Nagumo equation exhibits multiple, spatially uniform, steady states: one unstable and two stable (a lower one L and an upper one U). In such cases, the model caricatures bistable chemical systems and excitable membrane under special treatment. For an analytically solvable example, with diffusive transport in one space dimension, three types of traveling wave solutions, their propagation speeds, and dependence on model

parameters are described. Two waves correspond to the transitions from one stable steady state to the other. In addition, there may be a single stable pulse wave which represents an excursion from a stable steady state back to itself. By numerically integrating the full nonlinear reaction-diffusion equations we have studied interaction phenomena of waves with each other (e.g., annihilation) and with spatially localized reactant disturbances (e.g., reflection). A manuscript in collaboration with Dr. Terman is in preparation. (J. Rinzel).

Investigation continues of numerical methods for directly calculating periodic solutions to nonlinear oscillator problems. With R. Miller (paper to appear in Mathematical Biosciences) a finite difference method with a uniform mesh was developed and applied to calculate space-clamped periodic pulse train solutions to the HH equations. Currently with W. Troy (Univ. of Pittsburgh), a software package (COLLSYS) which uses a collocation method and adaptive non-uniform mesh selection is being tested on periodic solutions of the, rather stiff, Field-Noyes equations which model the Belousov-Zhabotinskii chemical reaction. (J. Rinzel).

In some models for reaction-diffusion systems, like certain of those for nerve conduction, a parameter variation changes the reaction dynamics from excitable to oscillatory. To understand, under such a parameter variation, the structure of solutions which describe propagation phenomena we consider a simple model with only one state variable, phase. The family of traveling wave solutions, parametrized by spatial period L, and their dispersion relation are described analytically, and numerically for a solvable example. We also derive asymptotically the simple phase model from a more general model in which the dynamics exhibit strong attraction to a pulse-like or periodic trajectory. A paper on this work, with G. B. Ermentrout, has been submitted to J. Math. Biology. (John Rinzel).

Some chemical reactions exhibit two-dimensional spatial patterns of wave-like activity in the form of rotating spirals and expanding targets. For a simple qualitative model, particular solutions have been obtained (Kopell and Howard, 1980) which are thought to correspond to the one-dimensional analog of such waves. Our numerical integration of the full reaction-diffusion system, with these solutions as initial conditions, suggests that some of these solutions, previously conjectured to be stable, are unstable and therefore not physically observable. A paper, with G. B. Ermentrout, has been submitted to J. Math. Biology. (John Rinzel).

The facilitated transport of oxygen by hemoglobin has been represented up to now by a model that assumes for the chemical kinetics the single step reaction $P + X \underset{k}{\overset{k'}{\rightleftharpoons}} PX$ where k′ and k are constants. We developed as reported previously, a numerical method for obtaining solutions for this model. A more correct representation would be the Adair four step binding model $PX_{i-1} + X \underset{k_D^i}{\overset{k_R^i}{\rightleftharpoons}} PX_i$, i=1, ..., 4. We showed that by a natural averaging the four step process can be mapped into a process formally similar to the single step reaction. The attending new overall k′ and k are some averages of the constants k_R^i and k_D^i

respectively. These average coefficients are no longer constants. We have then extended the numerical method to the case of nonconstant chemical coefficients. By using the values for the constants k_R^i and k_D^i reported in the literature we have shown that the facilitated diffusion by hemoglobin could be significantly larger than that obtained with the single step model. Further, the dissociation coefficients have been extrapolated to fit the oxygen dissociation curve for hemoglobin in the red cell. The values of facilitated oxygen transport obtained make it plausible that facilitation plays a role in the transport of oxygen in the systemic capillaries. (Jose M. Gonzalez-Fernandez and Susie E. Atta).

The way in which excitatory post synaptic potential (EPSP) time course and mechanism relate to the dendritic location of synapses was first made clear by a series of five collaborative papers in the Journal of Neurophysiology in 1967. We used shape index plots to compare the experimental EPSP time course with theoretical shape index loci deduced from the theoretical model. Because of recent refinements of data, examination of the shift of the theoretical shape index locus which results from shorter synaptic current duration and from different criteria for "rise time", was undertaken at the request of Drs. Sypert and Munson (at University of Florida College of Medicine, Gainesville). When this shifted theoretical locus was compared with their least square fit to their experimental shape index data, excellent agreement was found. Also, a very recent personal communication from Drs. Redman and Walmsley (at Monash University in Australia) reports that they have succeeded in the very difficult task of demonstrating agreement between synaptic location determined anatomically with horse radish peroxidase and the electrotonic distance of the same synapses deduced from EPSP shape indices and transient analysis. This provides beautiful confirmation of theoretical predictions which both they and we had made with our mathematical models. These and other recent results were presented and discussed with the several participants in a Symposium held at the XXVIII International Congress of Physiology in July 1980.

A theoretical basis for representing certain voltage transients (in dendritic neurons) as sums of exponential decays was provided by earlier studies (Rall, 1962, 1969) and tested on motoneurons of cat spinal cord. It was shown how estimation of the slowest decaying components of the experimental transients could provide information about the membrane time constant, the dendritic electronic length and the ratio of dendritic to somatic conductance and surface area. Very recently experimenters in several labs have begun to apply these methods to several different neuron types having different dendritic patterns. In response to this growing data and interest in transient analysis, new theoretical solutions have been obtained which yield explicit expressions for time constants and coefficients for cases where a neuron includes dendritic trees of significantly different electrotonic lengths. Computations with these solutions will provide further opportunities for comparisons with the variety of new data.

The theoretical model for extracellular field potentials generated by synchronous antidromic activity of a cortical population of neurons was presented and discussed in a mathematical neurobiology workshop held in Vancouver in June 1980. (Wilfrid Rall).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 13,000-07 MRB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mathematics of kinetics and reaction-transport systems.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John Z. Hearon Chief, MRB MRB, NIAMDD

COOPERATING UNITS (if any)

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SUMMARY OF WORK (200 words or less - underline keywords)

This project continues to focus upon applications of linear algebra, matrix and vector space methods to problems in biology. Applications are heavily involved in compartmental analysis; linear control theory, and generalized kinetics. No small part of the project is devoted to meeting purely mathematical problems which arise on their proper abstract grounds.

Project Number: Z01 AM 13,000-07 MRB

1. Extension of a Classical Theorem on Vector Norms.

In 1961, Bauer introduced the concepts of absolute and monotone vector norms. We call a norm, ϕ, absolute if it depends only on the absolute values of the entries of the vector. A norm ϕ is monotonic if $|x_i| \geq |y_i|$ implies $\phi(x) \geq \phi(y)$ where x_i and y_i are the entries of the vectors x and y. It is known that these properties are equivalent. Further it is is known that both of these properties are equivalent to the property that the matrix norm induced by ϕ is axis-oriented, i.e. $||D|| = \max|d_{ii}|$ for any diagonal matrix D where $||A||$ is the norm induced by ϕ. These properties are of general importance in linear algebra and of much practical value in numerical analysis. We have extended these results in the following

THEOREM:

Let N(A) be a generalized matrix norm (not necessarily induced by a vector norm). Then if N(A) is axis-oriented, any vector norm consistent with N is absolute and hence monotone.

This theorem includes the induced case since an induced matrix norm is necessarily consistent with the inducing vector norm. It states that axis-oriented matrix norms are consistent only with absolute vector norms, so in choosing a consistent pair of norms for any purpose if the matrix norm is axis-oriented we can confine ourselves to absolute vector norms. The proof is incredibly simple. Let N be a generalized norm such that $N(A) = N(A^{-1}) = 1$, for some A. Then A is a ϕ-isometry for any vector norm ϕ consistent with N. For, $\phi(Ax) \leq N(A)\ \phi(x) \leq \phi(x)$ and $\phi(x) = \phi(A^{-1}Ax) \leq \phi(Ax) \leq \phi(x)$ shows that $\phi(Ax) = \phi(x)$ for any x. Suppose now that N is axis-oriented. Then for $D = \text{diag}(d_1, d_2, d_3, \ldots, d_n)$ with $|d_i| = 1$, we have $N(D) = N(D^{-1}) = 1$. Thus any such D is a ϕ-isometry for ϕ consistent with N. But there exists for, arbitrary x, a D such that $Dx = |x|$ and we have $\phi(Dx) = \phi(x) = \phi(|x|)$ showing ϕ to be absolute. Note that we do not require a multiplicative matrix norm and that the $N(A) = N(A^{-1}) = 1$, N and ϕ consistent imply A is a ϕ-isometry was shown earlier as part of a more general study of norm-isometries. (Annual report F.Y. 1977).

2. Mean life and stationary content of compartmental systems.

On the basis of results reported last year we have proved the following. For any initial conditions let $\bar{t}_i$ be the mean life (residence time) of those particles initially in the i^{th} compartment of a compartmental system, irrespective of the sites of exit from the system. Let v be a constant input vector, otherwise arbitrary, with components v_i. Consider a steady-state system under input vector v and let the stationary amount in the system be S then we have the

THEOREM: In the steady-state under constant input v, the mean life is $\tau = \Sigma\bar{t}_i v_i / \Sigma v_i$. Further the stationary amount is $S = R\tau$ where R is the total rate of input Σv_i.

Note that the theorem states that τ is the weighted mean of the $\bar{t}_i$ with weights v_i/R which, are the fractional inputs to the individual compartments. This relation between stationary amount input rate and mean life has previously been derived for flow systems with single input and single exit. This is t first derivation for a compartmental system and is for the arbitrary multi-input multi-exit case. Given certain results the proof is rather short. We can show that $\bar{t}_i = -u^* A^{-1} u_i$ where A is the compartmental matrix, $u^* = [1, 1, \ldots, 1]$ and $u_i^* = [0, 0, \ldots 1, \ldots 0, 0]$ with 1 in the i^{th} position, and that $\tau = -u^* A^{-1} v/u^* v$. But $v = \Sigma u_i v_i$, thus τ is as stated in the theorem. Now the compartmental system with matrix A and input v obeys $\dot{x} = Ax + v$ where $x^* = [x_1, x_2, \ldots, x_n]$ with x_i the amount is the i^{th} compartment. As $t \to \infty$ we have $\dot{x} = 0$ and $x(\infty) = -A^{-1}v$. But $S = u^* x(\infty) = -u^* A^{-1} v = (-u^* A^{-1} v/u^* v) \cdot u^* v = \tau R$ since $u^* v = \Sigma v_i = R$. Nothing beyond the standard compartmental assumption has been used in this very general derivation.

3. Information Theory of Genetic Code

In a major paper on an information-theoretic treatment of the genetic code Tsukamoto (J. Theor. Biol. 78, 451 (1979)) seeks to minimize the information function $F = \Sigma p_i/q_i$, with fixed p_i and q_i subject to the constraint $\Sigma q_i = 1$. By partial differentiation he arrived at a correct relative extremum but gave a faulty proof that the extremum was a minimum . More important differential methods alone can establish only relative extrema. We have given a simple proof of the existence of an absolute minimum.

Let $f = \Sigma q_i - 1$ and consider $H = F + \lambda f$ where λ is a Lagrangian multiplier Then $\partial H/\partial q_i = -p_i/q_i^2 + \lambda = 0$, giving $\sqrt{\lambda} = \Sigma\sqrt{p_i}$, $q_i = \sqrt{p_i/\lambda}$ and, at the extremum, $F = (\Sigma\sqrt{p_i})^2$. Then by the Cauchy inequality $(\Sigma\sqrt{p_i})^2 \leq \Sigma p_i/q_i$, since $\Sigma q_i = 1$. Thus the extremum is a global minimum and this value is achieved at no other point in q-space by the known conditions for equality in the Cauchy inequality.

The derivation by Tsukamoto required three printed pages and failed to settle the global question.

Publications:

Hearon, J. Z.: A monotonicity theorem for compartmental systems. Math. Biosci. 46:293-300, 1979.

Hearon, J. Z.: On the completely symmetric compartmental system. Bull. Math. Biol. 42:481-488, 1980.

Hearon, J. Z. and Tsukamoto, Y.: Simple derivation of the function of genetic information and correction of the proof. J. Theor. Biol. 83, #3:525-526, 1980.

Kempner, E. S., Miller, J. H., Schlegel, W. and Hearon, J. Z.: The functional unit of polyenzymes. J. Biol. Chem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 13,001-07 MRB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mathematical formulations and analysis relevant to experimental neurophysiology.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Wilfrid Rall Senior Research Physicist MRB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

For several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models, relevant to experimental neurophysiology and neuroanatomy. Together these models provide a theory that can account for various sequences of events in the soma and dendritic branches of a single neuron, and for field potentials generated by certain cortical populations of neurons. Computational experiments performed with these models provide theoretical predictions that have been compared with experimental results obtained by colleagues with motoneurons of cat spinal cord, and with the mitral cell and granule cell populations of rabbit olfactory bulb. Resulting interpretations contribute to understanding of dendritic synaptic input and of dendro-dendritic synaptic interactions. These results have appeared in the Biophysical Journal and in the Journal of Neurophysiology. A comprehensive chapter, entitled "Core conductor theory and cable properties of neurons" (in The Handbook of Physiology: The Nervous System, Vol. I, Cellular Biology of Neurons, American Physiological Society 1977) summarizes some of these results.

PHS-6040

Project Number: Z01 AM 13,001-07 MRB

1. The way in which excitatory post synaptic potential (EPSP) time course and mechanism relate to the dendritic location of synapses was first made clear by a series of five collaborative papers in the Journal of Neurophysiology in 1967. We used shape index plots to compare the experimental EPSP time course with theoretical shape index loci deduced from my theoretical model. Because of recent refinements of data, Drs. Sypert and Munson (at Univ. of Florida College of Medicine, Gainesville) asked me to examine the shift of the theoretical shape index locus which results from shorter synaptic current duration and from different criteria for "rise time". When this shifted theoretical locus was compared with their least square fit of a parabola to their experimental shape index data, excellent agreement was found. Also, a very recent personal communication from Drs. Redman and Walmsley (at Monash Univ. in Australia) reports that they have succeeded in the very difficult task of demonstrating agreement between synaptic location determined anatomicall with horse radish peroxidase and the electrotonic distance of the same synapses deduced from EPSP shape indices and transient analysis. This provides beautiful confirmation of theoretical predictions which both they and we had made with our mathematical models. These and other recent results were presented and discussed with the several participants in a Symposium held at the XXVIII International Congress of Physiology in July 1980.

2. A theoretical basis for representing certain voltage transients (in dendritic neurons) as sums of exponential decays was provided by earlier studies (Rall, 1962, 1969) and tested on motoneurons of cat spinal cord. It was shown how peeling the slowest decaying components of the experimental transients could provide information about the membrane time constant, the dendritic electronic length and the ratio of dendritic to somatic conductance and surface area. Very recently experimenters in several labs have begun to apply these methods to several different neuron types having different dendritic patterns. In response to this growing data and interest in transient analysis, I have recently obtained new theoretical solutions which yield explicit expressions for time constants and coefficients for cases where a neuron includes dendritic trees of significantly different electrotonic lengths. Computations with these solutions will provide further opportunities for comparisons with the variety of new data.

3. The theoretical model for extracellular field potentials generated by synchronous antidromic activity of a cortical population of neurons was presented and discussed in a mathematical neurobiology workshop held in Vancouver in June 1980.

Publications:

Rall, W.: Functional aspects of neuronal geometry, in Neurons without Impulses (Roberts and Bush, eds.), Cambridge Univ. Press (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 13,002-08 MRB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mathematical description of the substrates transport in the capillary-tissue structures.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jose M. Gonzalez-Fernandez	Research Mathematician	MRB, NIAMDD
OTHER:	Susie E. Atta	Mathematician	MRB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	2.0	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To develop mathematical models of the blood flow and transcapillary exchanges in capillary networks. An effort is being made to incorporate in the models the histological structure of capillary networks as well as different flow patterns from available experimental information. In this model the extraction of substrates with different chemical kinetics at the tissue site will be described. It is expected that this could be used in experimental situations where the extraction of different substrates are measured simultaneously, thus helping to infer the flow pattern features of the microcirculation. In particular a model of the diffusion-consumption of oxygen in striated muscle containing myoglobin (facilitated diffusion) is being developed and pertinent numerical results examined.

PHS-6040

Project Number: Z01 AM 13,002-08 MRB

The facilitated transport of oxygen by hemoglobin or myoglobin has been represented in the literature by the diffusion of and the chemical interaction between the substrates oxygen, protein and the compound protein oxygen. The chemical kinetics involved were described by the single step reaction

$P + X \underset{k}{\overset{k'}{\rightleftharpoons}} PX$ where k′ and k are constants. We developed, as reported previously, a general numerical method for obtaining solutions for this model.

For the chemical reaction of hemoglobin with oxygen a more correct representation than the one mentioned above would be the Adair four step binding model $PX_{i-1} + X \underset{k_D^i}{\overset{k_R^i}{\rightleftharpoons}} PX_i$, i=1, ..., 4. We show that by a natural averaging this four step process can be mapped into a process formally similar to the single step reaction. The attending new overall recombination k′ and dissociation k coefficients are some averages of the constants k_R^i and k_D^i i=1, ..., 4 respectively. Those average coefficients are not constants. We have extended the numerical method to the case of nonconstant chemical coefficients. By using the values of the constants k_D^i, k_R^i available in the literature we showed that the facilitated transport by hemoglobin in solution could be significantly larger than the one calculated by the single step model. This difference in facilitation is largely related to the change in the associated oxygen dissociation curve from a hyperbola to a sigmoid.

It has been shown that the naturally occurring 2, 3-diphosphoglycerate in the red cell decreases the affinity of the hemoglobin for oxygen and that this is effected by an increment on the value of the dissociation constants. For the hemoglobin in the red cell, p_{50} = 24 torr, we have shown that these effects enhance the facilitation by promoting the dissociation of oxygen in the vicinity of the low oxygen concentration boundary. By using values for the boundary conditions motivated by the partial pressures of oxygen found along the systemic capillaries we have shown that for a slab one micron thick the facilitated flow contributes 50 to 65% of the total transport. One micron is likely to be within the order of magnitude of the diffusion path in the red cell inside the capillary. This suggests the possibility that facilitated flow may contribute to the transport of oxygen in the systemic capillaries.

A result of independent interest obtained here is that the values reported in the literature for the chemical kinetic constants appearing in the single step model representation yield a lower bound for the speed of the chemical kinetics of hemoglobin with oxygen. (Jose M. Gonzalez-Fernandez and Susie E. Atta).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 13,004-06 MRB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Mathematical description of cellular neuroelectric signal transmission

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John Rinzel	Senior Scientist	MRB, NIAMDD
OTHER:	G. Bard Ermentrout	Staff Fellow	MRB, NIAMDD
	David Terman	Summer Employee	MRB, NIAMDD
	Randall LeVeque	Summer Employee	TBS, NHLBI

COOPERATING UNITS (if any)
R. Miller, Post-doc Scientist
Harvard University
Cambridge, MA

W. C. Troy, Assoc. Prof/Math.
University of Pittsburgh
Pittsburgh, PA

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.3	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of neuroelectric signaling for individual neurons. Among the topics of current interest are:

(i) integration of synaptic input delivered to the soma and dendritic branches of a neuron;

(ii) steady propagation of action potentials along axons;

(iii) stimulus-response and threshold properties for repetitive firing of action potentials;

Mathematical models of these phenomena involve systems of linear and nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods. One aspect in the approach of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet reasonable, equations. Because qualitatively related mathematical problems arise in other biological and chemical contexts, e.g. chemical and biochemical oscillations, this project may consider models from such applications.

Project Number: Z01 AM 13,004-06 MRB

1. (With Robert Miller) The speed of a propagating nerve impulse depends on the level of refractoriness in the wake of its predecessor. Hence, in general, speed and interspike interval (ISI) change during propagation. This project has emphasized that a qualitative understanding of this phenomenon may be inferred from the dispersion relation (speed versus ISI) for steadily propagating periodic wave train solutions of nerve conduction equations. Recently we have shown that this relation carries quantitative significance. By interpreting ISI in the dispersion relation as instantaneous interspike interval we derive an approximate kinematic description for the changing speeds an ISI's of individual pulses during propagation. For the Hodgkin-Huxley (HH) model, comparisons are made between pulse trajectories which are computed easily by kinematics with those obtained from numerical solutions of the full HH cable equations. Such comparisons verify empirically that the approximate description is quantitatively accurate over a substantial range of ISI's. From the kinematic description one predicts analytically that a second pulse may lock into a fixed interval and travel at the same speed as its predecessor if the dispersion relation exhibits a range of ISI's with supernormal speeds. Some experimental evidence (e.g., Waxman and colleagues) suggests that such locking is associated with supernormal conduction and occurs in mammalian CNS axons. In a paper submitted to the Biophysical Journal, our kinematic results and dispersion calculations, including observations of supernormal speeds, for the HH model are described and related to experiments.

Kinematic pulse trajectories, for a simplified FitzHugh-Nagumo nerve conduction model, were also found to compare accurately with actual theoretical pulse trajectories even for relating short ISI's. For some parameter values, the dispersion relation is strikingly non-monotone with a prominent supernormal speed hump (where the speed for some ISI's exceeds the solitary pulse speed by 50%). Kinematic calculations illustrate how a stimulus train with uniform interstimulus intervals in the supernormal range may yield a train of pulses which, during propagation, form locked pairs of alternating short and longer ISI's. In contrast, if the dispersion relation is primarily monotone (speed increasing with ISI) as for the HH model at 6.3°C then ISI's tend toward uniformity during propagation. Such examples illustrate qualitative differences in signaling characteristics for a general class of systems with either monotone or non-monotone dispersion relations. (J. Rinzel, Impulse propagation in excitable systems. Proc. of Mathematics Research Center Advanced Seminar: Dynamics and Modelling of Reactive Systems. 1980).

2. (With D. Terman) For some parameter ranges, the FitzHugh-Nagumo equation exhibits not just one but three spatially uniform steady states: one unstable and two stable (a lower one L and an upper one U). In such cases, the model caricatures bistable chemical systems and electronic transmission lines, and excitable membrane under special treatment. We consider this dynamics with diffusive transport in one space dimension and find that a nonuniform initial condition may evolve into a traveling wave profile. For a simplified equation with piece-wise linear dynamics, we have obtained analytically three types of traveling wave solutions, their propagation speeds, and dependence on model parameters. Two waves correspond to the transitions U to L and L to U; these have speeds c_- and c_+, respectively. In addition, there may be a single stable pulse wave which represents an excursion from a stable steady state, either U

or L, back to itself. An L to L pulse occurs only if $c_- > c_+$ or a U to U pulse occurs only if $c_+ > c_-$. By numerically integrating the full nonlinear reaction-diffusion equations we have studied the interaction of the waves with each other and with spatially localized reactant disburbances. For example, a rightward moving L to U transition may, upon encountering a region where the reactants are biased away from U, reverse itself and become a leftward moving U to L transition. Our numerical experiments suggest that this can happen only if $c_- > c_+$. Moreover, in such cases, we have been unsuccessful in trying to reverse a U to L transition. A manuscript which describes these results is in preparation.

3. Investigation continues of numerical methods for directly calculating periodic solutions to nonlinear oscillator problems. The methods under consideration seek periodic orbits as solutions to two-point boundary-value problems with periodic boundary conditions. With R. Miller (paperin Math. Biosc.) a finite difference method was developed and applied to calculate space-clamped as well as steadily propagating periodic pulse train solutions to the HH equations. The method uses stiffly stable backward difference formulae, of up to fifth order, with a uniform mesh. Subsequently, with R. LeVeque, a generally available computer code PASVA, was implemented, tested, and found competitive. It uses a second-order centered difference scheme with nonuniform adaptive mesh selection and deferred correction to achieve higher order convergence. Currently with W. Troy, a collocation method with spline basis functions and adaptive nonuniform mesh is being tested on the limit cycle solutions of the, rather stiff, Field-Noyes equations which model oscillations of the Belousov-Zhabotinskii chemical reaction.

4. (With B. Ermentrout) In some models for reaction-diffusion systems, like certain of those for nerve conduction, a parameter variation changes the reaction dynamics from excitable to oscillatory. We seek to understand, under such a parameter variation, the structure of solutions which describe propagation phenomena. For a simple model with only one state variable, phase, we qualitatively examine this structure. There is a family of traveling wave solutions parameterized by spatial period L. These solutions and their dispersion relation are described analytically, and numerically for a solvable example. In the excitable regime, the solution for $L = \infty$ corresponds to a solitary pulse with finite speed. In the oscillatory regime, as $L \to \infty$, the speed becomes infinite and the spatially homogeneous oscillation is obtained. We also derive asymptotically the simple phase model from a more general model in which the dynamics exhibit strong attraction to a pulse-like or periodic trajectory. (Paper submitted to J. Math. Biology).

5. (With B. Ermentrout) The Belousov-Zhabotinskii chemical reaction can exhibit two-dimensional spatial patterns of wave-like activity in the form of rotating spirals and expanding targets. For a simple qualitative model, particular solutions have been obtained (Kopell and Howard, 1980) which are thought to correspond to the one-dimensional analog of such waves. Our numerical integration of the full reaction-diffusion system, with these solutions as initial conditions, suggests that some of these solutions, previously conjectured to be stable, are unstable and therefore not physically observable. (Paper submitted to J. Math. Biology).

Project Number: Z01 AM 13,004-06 MRB

Publications:

1. Rinzel, J.: Impulse propagation in excitable systems, in Dynamics and Modelling of Reactive Systems (W. E. Steward, W. H. Ray, and C. C. Conley, eds.), Academic Press (1980).

2. Guttman, R., S. Lewis, and J. Rinzel: Control of repetitive firing in squid axon membrane as a model for a neuron oscillator. J. Physiol. (Lond.), in press.

3. Rinzel, J. and R. Miller: Numerical calculation of stable and unstable periodic solutions to the Hodgkin-Huxley equations. Math. Biosc., 49: 27-59, 1980.

4. Ermentrout, G. B. and J. Rinzel: One dimensional λ-ω target patterns: empirical stability tests. J. Math. Biol. (in press).

5. Ermentrout, G.B. and J. Rinzel: Waves in a simple excitable or oscillatory reaction-diffusion model. J. Math. Biol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 13,013-01 MRB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mathematical modeling of biological waves.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Bard Ermentrout	Staff Fellow	MRB NIAMDD
OTHER:	John Rinzel	Senior Scientist	MRB NIAMDD

COOPERATING UNITS (if any)
Jack D. Cowan, Professor
Department of Biophysics
University of Chicago

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.3	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project focuses on the formulation, analysis, and interpretation of mathematical models which describe biological and chemical waves. The main topics of interest are:

(i) neuronal networks and their application to the understanding of pathological neural phenomena such as epilepsy and hallucinations;

(ii) excitable systems capable of producing pulse-like responses to appropriate stimuli;

(iii) chemical oscillators, spiral waves, and coupled oscillations;

(iv) stationary, homogeneous media, a class of very general physical and biological models;

(v) nonlinear stability theory, bifurcation theory, as applied to physiological systems.

Our prime objective is to expose the qualitative behavior of the models as an explanation for observed biological patterns.

PHS-6040

Project Number: ZO1 AM 13,013-01 MRB

1. (With Jack D. Cowan) We have analyzed a simple model for a two layer network of excitatory and inhibitory neurons. In previous work we described certain oscillations and waves which we used as a model for electroencephalographic patterns. We have continued this investigation and have attempted to describe, in terms of the model, the basic mechanisms underlying experimental epilepsy. In particular, we have determined various qualitative properties of epileptic waves such as dispersion, frequency, and amplitude and compared them to the model.

We are continuing an investigation of the mechanisms underlying visual hallucinations. In a previous paper we solved a simple model which explained the four form constants described in the experimental literature. We are in the process of refining this work in order to make it more physiologically plausible.

2. (With John Rinzel) We have proposed a simple caricature for an excitable and/or oscillatory system. This device is a first order nonlinear differential equation which depends on a parameter, γ. As γ varies the qualitative behavior changes from oscillatory to excitable. When many of these devices are diffusively coupled on a physical ring, various wave forms can result. In the oscillatory case, we construct a one parameter family of "periodic" wavetrains with a well-defined dispersion relation. These waves have many of the same properties as those found on more complicated systems. For the excitable case, we construct a "solitary" pulse wave and also a one-parameter family of wavetrains.

We then show how this simple caricature arises in a natural fashion from more complicated problems. An existence theorem for pulse waves in general systems is proven using a singular perturbation argument. We reduce the general system to the caricature described above and use the results about that system to describe the general model. These results have been submitted for publication to J. Mathematical Biology.

3. (With John Rinzel) Recently investigators have proven an existence theorem for "one dimensional spiral and target waves" in a simple chemical reaction system. We studied the stability of these solutions using numerical methods. We found that in all cases, these waves were unstable and were able to describe the mechanism underlying the instability. These models arose as an attempt to explain certain patterns experimentally observed in oscillating chemical reactions. That the mathematical patterns appear to be unstable casts doubt on this particular mechanism. This work has been accepted for publication in J. Math. Biol.

4. There has recently been some controversy as to whether stable small amplitude solutions exist for reaction-diffusion models. We have partially resolved that question by describing a two-parameter family of stable small-amplitude wavetrains. A two parameter Poincare-Lindstedt series is developed near a bifurcation point. Various nonlinear stability theorems are used to describe the rate of decay of perturbations about the above solution. A range of values for the various parameters is found within which there are stable periodic waves. An alternative method which uses multiple scales is applied to show the existence of stable long waves. A short version of this paper has been published in the proceedings of a conference.

The complete version has been submitted to the Quarterly of Applied Mathematics.

5. Many biological phenomena can be described by models which are invariant under translations in time and space - that is they are homogeneous and stationary. I have developed a theory for describing the possible nontrivial patterns which can arise in these models as the rest state loses stability. A method for multiple scales in general nonlinear problems is developed and applied to these models. Various waves and spatial patterns are possible and a method is derived for establishing their stability. The results of this investigation are in "Stationary, homogeneous media: I. One dimensional isotropic media" to be submitted to Physica D. Another manuscript which deals with the anisotropic case in one and two dimensions is in preparation.

Publications:

Ermentrout, G. B.: Small amplitude stable wavetrains in reaction-diffusion systems. To appear in: Proceedings of the Conference on Nonlinear Partial Differential Equations in Engineering and Applied Sciences.

Ermentrout, G. B. and Rinzel, J.: One dimensional λ-ω target patterns: empirical stability tests. J. Math. Biol. (in press).

Ermentrout, G. B.: Stable small amplitude solutions in reaction-diffusion systems. Quart. Appl. Math. (in press).

Ermentrout, G. B. and Rinzel, J.: Waves in a simple excitable or oscillatory, reaction-diffusion model. J. Math. Biol. (in press).

Annual Report of the Arthritis and Rheumatism Branch

National Institute of Arthritis, Metabolism and Digestive Diseases

The Branch has continued to emphasize studies bearing upon systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), the former relying heavily upon a clinical and laboratory data base which is computerized (ZO1 AM 41029).

Genetic (ZO1 AM 41037) and epidemiologic (ZO1 AM 41036) aspects of SLE have been investigated. It was found that certain B-lymphocyte alloantigens, controlled by genes of the major histocompatibility complex are present in increased frequency in SLE. Family studies strongly imply that coinheritance of several of these antigens augments a person's susceptibility to the disease. No connection between canine SLE and the human disease was detected in a detailed study of household contacts recently reported in full.

The major therapeutic studies in reference to SLE nephritis continue (ZO1 AM 41000 & ZO1 AM 41040). Our most recent data summary as of December 1979 shows that patients receiving certain forms of immunosuppressive drugs with prednisone (designated "cytotoxic" group) do better in some respects than patients receiving only prednisone (designated "prednisone" group). Decline in renal function was observed in 3/27 (11%) of cytotoxic and 4/12 (33%) of prednisone, a trend regarded as encouraging. The data showed many more flare-ups of systemic disease in the prednisone group (13 in 39 patient years) than in the cytotoxic (6 in 51 patient years). Prednisone dosage was higher and complications ascribed to corticosteroids more numerous in the prednisone only group.

Ibuprofen, compared in a blind study to aspirin, was found to be slightly less effective in controlling minor features of SLE (ZO1 AM 41041). Pneumococcal vaccine safely produced substantial antibody titers in SLE patients, but the titers were generally lower than in normals (ZO1 AM 41043). The role of renal histology as a predictor of outcome in SLE nephritis is under active study using a semi-quantitative histological scoring system (ZO1 AM 41038).

The control and modulation of the complex immunological network seem to be disordered in SLE. A number of recently defined elements of this disorder have been shown to be related to disease activity, that is, present and playing a role in active disease and absent or much reduced in quiescent disease (ZO1 AM 41023 and ZO1 AM 41052). This is true of immunoglobulin secreting B-lymphocytes, of suppressor T-lymphocyte function as well as suppressor cell precursor numbers, and of circulating anti-T cell antibodies, antibodies capable of antibody-dependent cytotoxicity.

A remarkable proportion of the above findings in humans were earlier detected or predicted based upon the autoimmune mouse model of the disease which continues under investigation (ZO1 AM 41020, ZO1 AM 41021, ZO1 AM 41022). The major recent emphasis has been on the genetic background and upon the role of sex hormones in the expression of the disease. The genetic work supports the concept of separate and independent T cell and B cell

abnormalities. Sex hormone manipulation does not appear to reverse the disease process; rather it has transient effects, particularly notable early in the life of the animal.

RA has been another nidus of Branch interest. A detailed, double-blind study of 12 severely ill RA patients has been completed; 6 were randomized to sham lymphapheresis and 6 had bona fide lymphapheresis carried out (ZO1 AM 41044). There was a clear and significant advantage to those receiving lymphapheresis with distinct recession of disease activity. However the degree of improvement observed was far less than was achieved in an earlier open study of the identical procedure (ZO1 AM 41042). Attempts to determine which of the mononuclear cells removed is the pertinent one are underway.

A pilot study of bolus corticosteroids (1 gm methylprednisolone iv daily for 3 days) for severe RA showed impressive immediate benefit to all and prolonged benefit to a majority (ZO1 AM 41053). This is to be followed up with pharmacologic studies of the same dose comparing the intravenous to the per oral route of administration. A study of repeated intravenous cyclophosphamide for severe RA continues to admit patients (ZO1 AM 41042). In addition the Branch, primarily on an outpatient basis, has been contributing patient observations to two national cooperative studies of RA under the aegis of the Center for Cooperating Studies in the Rheumatic Diseases (ZO1 AM 41049, ZO1 AM 41050).

A new area of interest is in the extraordinarily persistent synovitis which appears in rats after a single intraperitoneal injection of cell walls from Group A streptococci (ZO1 AM 41046). Striking divergences in the prevalence of arthritis after such injections have been found among selected inbred strains; in some, every animal developes widespread polyarthritis while in others no arthritis whatsoever appears. Efforts are underway to establish the phenotypic factors which are responsible for this divergent reaction to the foreign material; they are in part, at least, under genetic control.

The model immune complex studies have demonstrated the profound effects of the chemical nature of the antigen (ZO1 AM 41018). In the case of carbohydrate containing antigens, the exposed sugar moieties of the immune complex have a major impact on the complex clearance rate and on the clearing cell. For example, an immune complex containing orosomucoid, a glycoprotein, was taken up relatively slowly by the Kupfer cells whereas an identical complex containing asialo-orosomucoid with exposed galactose moieties is taken up very rapidly by the hepatic parenchymal cells themselves. Ficoll, a sucrose polymer, was used as an antigen with adducts of either mannose or galactose, the modified Ficoll was covalently linked to IgG and clearance of the model complex was assessed. Mannose-bearing complexes were taken up by the Kupfer cells and galactose-bearing by the hepatocytes.

The cell surface location of these receptors was suggested by the displacement of recently cleared model immune complexes back into the circulation of NZB/W mice upon treatment with large doses of aggregated human gamma globulin. Similarly presaturation with uncomplexed asialoceruloplasmin greatly inhibits hepatocyte uptake of complexes containing asialo-orosomucoid.

Progress was made in the study of the IgE receptor system (ZO1 AM 41025). Of interest in its own right, the system is also considered a model of antigen-induced antibody-mediated cellular actions. The receptor now appears to include two polypeptide chains, dubbed α and β, where only one was earlier recognized. The second chain, β, isolated under very mild conditions, has the properties of a subunit rather than simply a chain cross-linked to the α chain. The size of the functional unit, determined by assessing activity after exposure to ionizing radiation, target analysis, actually suggests that the unit may be smaller than the α chain. The β chain was isolated cross-linked to the α chain and became apparent with the use of the photolyzable reagent, iodonaphthylazide.

Other work has shown that receptor bound IgE is far less subject to catabolism than is the circulating immunoglobulin (ZO1 AM 41051). Characterization of the receptor activity of several cloned sublines of rat basophilic leukemia cells suggest that the differences in histamine releasing capacity are not dependent upon differences at the receptor level; the latter functioned evenly in all sublines.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41000-11 ARB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Controlled study of cyclophosphamide and azathioprine in systemic lupus erythematosus with nephritis (CAP)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.L. Decker	Chief, Arthritis and Rheumatism Br.	ARB NIAMDD
	A.D. Steinberg	Senior Investigator	ARB NIAMDD
OTHER:	P.H. Plotz	Senior Investigator	ARB NIAMDD
	J.H. Klippel	Senior Investigator	ARB NIAMDD
	J.E. Balow	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with diffuse glomerulonephritis and systemic lupus erythematosus satisfying criteria for active renal disease are offered a therapeutic study. The patients are randomized to different treatment programs which include the following drugs: corticosteroids, azathioprine and cyclophosphamide.

Follow-up visits occur every six months and the patients are followed until death or dialysis. Clinical and immunologic assessment are performed. The risks and benefits of each treatment will be evaluated and compared.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Two ten week studies by our Branch suggested that cyclophosphamide was effective in the treatment of lupus nephritis and that azathioprine was less effective. A long-term study of these patients is being carried out to determine whether or not one therapeutic program is superior over the course of several years.

Methods Employed:

Patients meeting predefined criteria for diagnosis of SLE and activity of nephritis have been admitted to the trial. In a baseline control period, the patients were kept on the anti-inflammatory regimen (usually prednisone and aspirin) needed to control extra-renal manifestations. These have been continued with as little change as possible.

A large number of serological, chemical and urine values are serially obtained and the patients will be followed for life. Immunoglobulin levels, anti-DNA antibodies, and serum complement are among those measures. We will determine time to death or renal dialysis in each treatment program and assess the toxicity as well as efficacy of each program.

Major Findings:

The first patient was admitted to this trial in April 1970 and the 38th and last in October 1972.

After courses ranging up to 7 years duration, we were unable to detect an advantage conferred by cyclophosphamide or azathioprine to corticosteroids alone. Furthermore, we were increasingly impressed with the untoward effects of cyclophosphamide, particularly hemorrhagic cystitis and carcinoma of the bladder. In contrast, azathioprine appeared quite safe.

Accordingly, in March 1979 a plan was developed for the discontinuation of these cytotoxic drugs and the intensive observation of study patients during the period following withdrawal. All patients were withdrawn from either cyclophosphamide or azathioprine by December 1979. No obvious exacerbations of SLE were detected as result of drug withdrawal.

Significance to Bio-Medical Research and the Program of the Institute:

This work has already had wide medical impact. The project should allow us to draw conclusions in reference to the long-term hazards of drug administration and withdrawal.

Publications:

Information gained in this study has been reported in references cited with Z01 AM 41040-07.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41001-09 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Therapy of SLE: A controlled trial comparing high dose intravenous cyclophosphamide with placebo.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PROJECT TERMINATED

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41018-06 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Production of covalently cross-linked immune complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	D. Finbloom	Clinical Associate	ARB NIAMDD
	D. Magilavy	Clinical Associate	ARB NIAMDD
	A. Rifai	Staff Fellow	ARB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Arthritis and Rheumatism Branch

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Three major aspects of immune complex biology have been been studied. First, the ability of the mouse liver to handle infused complexes was studied, using site-specifically cross-linked model complexes. The receptors for complexes on Kupffer cells were found to be specific for the immunoglobulin molecule and not other aggregated proteins removed by the same cells. Furthermore, NZB/W mice, with murine lupus, show an impaired ability to injest complexes after they are bound to Kupffer cells. Second, the influence of antigen or the handling of complexes has been studied using pairs of glycoprotein antigens differing only in their carbohydrate side chains but reacting with the same antibodies to form complexes. Desialylated glycoproteins carry complexes to hepatocytes, whereas complexes with the intact protein go to Kupffer cells. Third, the influence of monosaccharides substituted onto the Ficoll backbone of another group site-specifically cross-linking antigens has been studied. Galactose substitution on the antigen carries the stable complexes to hepatocytes, whereas mannose brings them to Kupffer cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To study the role of the configuration of immune complexes in determining their pathogenetic properties.

Methods Employed:

Model complexes were made using a family of bivalent or multivalent affinity-labelling antigens. Immune complex handling was studied by in vivo injection or portal vein infusion of complexes. Kupffer and hepatocyte separation were carried out by enzymatic digestion of the liver.

Major Findings:

1. There are immunoglobulin-specific receptors on mouse Kupffer cells.

2. The behavior of these receptors is abnormal in murine lupus.

3. The nature of the antigen in an immune complex may have a profound influence on the handling of the complex.

Significance to Bio-Medical Research and the Program of the Institute:

The recognition that the handling of immune complexes may be influenced by the antigen in the complex suggests that the pathogenesis of immune complex disease may be partially under the influence of the antigens in the immune complexes.

Proposed Course:

The principal investigator will be on foreign assignment in England during the coming year, but aspects of the work will be continued by Dr. David Finbloom in Dr. Metzger's laboratory, Dr. Rifai at the University of Washington in Seattle, and Dr. Magilavy at Children's Hospital.

Publications:

1. Plotz, P.H., Kimberly, R.P., Guyer, R.L., and Segal, D.M.: Stable model immune complexes produced by bivalent affinity-labeling haptens: In vivo survival. Molecular Immunol. 16: 721-729, 1979.

2. Segal, D.M., Guyer, R.L., and Plotz, P.H.: Complement fixation by model immune complexes free in solution and bound onto cell surfaces. Biochemistry 18: 1830-1835, 1979.

3. Jones, J.F., Plotz, P.H., and Segal, D.M.: Complement and cell-mediated lysis of haptenated erythrocytes sensitized with oligomers of rabbit IgG antibody. Molecular Immunol. 16: 889-897, 1979.

4. Finbloom, D.S., and Plotz, P.H. : Studies of reticuloendothelial function in the mouse using model immune complexes. I. Serum clearance and tissue uptake in normal C3H mice. J. Immunol 123: 1594-1599, 1979.

5. Finbloom, D.S. and Plotz, P.H. : Studies of reticuloendothelial function in the mouse using model immune complexes. II. Serum clearance, tissue uptake, and reticuloendothelial saturation in NZB/W mice. J. Immunol. 123: 1600-1603, 1979.

6. Finbloom, D.S., Abeles, D., Rifai, A., Plotz, P.H. : The specificity of uptake of model immune complexes and other protein aggregates by the murine reticuloendothelial system. J. Immunol. 1980 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41020-13 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Pathogenesis of autoimmunity in mice with SLE-like illness

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D. Steinberg	Senior Investigator	ARB/NIAMDD
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	J. Taurog	Clinical Associate	ARB/NIAMDD
	P. Smathers	Biologist	ARB/NIAMDD
	J. Reeves	Chemist	ARB/NIAMDD
	E. Raveche	Chemist	LEP/NIAMDD
	J. Cowdery	Clinical Associate	ARB/NIAMDD
	E. Novotny	Animal Caretaker	ARB/NIAMDD
	C. Rubin	Staff Fellow	ARB/NIAMDD
	C. Hansen	Geneticist	R/VR
	J. Roths		The Jackson Laboratory, Bar Harbor, ME

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Small Animal Section, VR
The Jackson Laboratory, Bar Harbor, Maine

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Autoimmunity in New Zealand mice has a genetic and immune basis; however, expression of severity can be modified by viral and other environmental factors as well as sex hormones. The immunologic factors are complex. These include excessive B-cell activation and abnormal T cell function. Excessive B-cell activation can be modified by introducing the X chromosome of a CBA/N mouse into the NZB autoimmune background. The CBA/N X chromosome, which codes for defects in B-cell maturation (xid), causes a reduction in autoimmunity in F_1 mice, and in congenic mice. The B cell subset absent in CBA/N mice is that which is hyperactive in NZB mice. In contrast, MRL/l mice produce autoantibodies via a different B cell subset. Administration of polyclonal B-cell activators (poly rI·rC and LPS) bypasses the xid in NZB hybrids in that other subsets of B cells are stimulated to produce autoantibodies.

PHS-6040
(Rev. 10-76)

Project Description:

Objective:

New Zealand mice spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus. Early in life they are immunologically hyperactive, become relatively resistant to tolerance induction, and gradually develop antinuclear and other autoantibodies followed by clinical disease. Both humoral and cellular immune defects have been reported and are under active investigation. In addition, the bases for the differences in disease expression in New Zealand mice are being explored. Recently developed MRL/MP-lpr/lpr (MRL/l) and BSXB mice which develop SLE are also being studied.

Methods Employed:

1. Assays for antibodies to RNA and DNA using ^{14}C-labeled or ^{3}H-labeled nucleic acids as ligands and the ammonium sulfate precipitation assay. Assay of antibody forming cells to sheep erythrocytes and pneumococcal polysaccharide by local hemolysis in gel.

2. Immunization of animals with a variety of antigens with and without protein carriers or adjuvants.

3. Assay of antibodies to erythrocytes by passive hemagglutination.

4. Assay of antibodies to DNP-Ficoll by passive hemagglutination with TNP conjugated OX erythrocytes.

5. Assay of antibodies to bovine gamma globulin by solid phase radioimmunoassay.

6. Assay of IgM and total anti-DNA and anti-RNA by solid phase radioimmunoassay.

7. Assay of anti-idiotype antibodies to anti-rI·rC by solid phase radioimmunoassay.

8. Neonatal thymectomy by midline incision and a suction catheter.

9. Transfer of spleen, bone marrow and thymus cells into recipients to evaluate the functional properties of the different cell types.

10. Study of the graft versus host disease inducing properties of NZB lymphoid cells using the Simonsen spleen weight assay and newborn SWISS mice as recipients.

11. Separation of lymphoid cells functionally (corticosteroid, anti-T cell sera, PNA and physically (sedimentation).

12. Assay of anti-T cell antibodies by release of ^{51}Cr from thymocytes.

13. Evaluation of subpopulations of T-cells using the fluorescence activated cell sorter.

14. Castration of male and female NZB/W mice at various ages and treatment with sex hormones by either injection of implantation of silastic tubes containing crystaline hormones.

15. Immunization of mice with H-2 incompatible and H-2 compatible cells and studying in vitro regulation of T-cell function: proliferation in response to, and killing of, the H-2 incompatible cells as well as killing of additional target cells. These studies include time course of responsiveness and kinetics of killing during the early phase of the 4-hour cytotoxicity assay (using ^{51}Cr-labeled targets).

Major Findings:

1. Susceptibility to spontaneous anti-DNA is inherited as a single trait which is expressed in a gene dosage fashion. This is not linked to production of anti-T-cell antibodies which is controlled by a single trait and is expressed in a gene dosage fashion.

2. The cytotoxic lymphocyte responses of NZB mice were suppressed by allosensitized spleen cells but not by suppressor factors elaborated by such cells. This defect was found to be a recessive trait. It appears to be related to excessive differentiation and activation of NZB cells destined to become cytotoxic cells or to help such cells.

3. Kinetic analyses of NZB cytotoxic responses suggest that NZB mice respond to primary sensitization with an augmented primary response. They have a peak cytotoxic response on Day 4 as compared with Day 6 for control strains. The linear phase of killing during the 4 hour assay persists for a greater period of time than in control strains. Nevertheless, cells from primed control strains have even more accelerated killing than do non-primed NZB cells.

4. NZB mice and their F_1 hybrids produce excessive polyclonal IgM and autoantibodies of both IgM and IgG classes. CBA/N mice and CBA/N mothered F_1 males fail to make antibody to many T-independent antigens and have low levels of serum IgM; further, these mice lack a population of splenic B-cells characterized by a low-to-intermediate density of surface IgM. The CBA/N X-linked defect of T-independent immune response is completely expressed in CBA/N X NZB mice. In marked contrast to NZB mice and to NZB F_1 hybrids bearing at least one normal X-chromosome, the CBA/N x NZB males failed to respond to two T-independent antigens, had small numbers of splenic IgM producing cells, barely detectible splenic IgM production, and splenic B-cell surface immunoglobulin patterns resembling those of CBA/N mice. Such CBA/N X-chromosome bearing NZB mice fail to make anti-DNA. In contrast, MRL/1 mice bearing the CBA/N X-chromosome make anti-DNA. Congenic NZB mice are free of illness, whereas congenic MRL/1 mice are ill.

5. Thymectomy at birth retards the autoimmune process of MRL/1 mice.

6. In NZB mice, B cells are responsible for the high IgM production, T cells for tolerance defects, and both are necessary for maximum autoantibody production.

Proposed Course:

The role of genetic factors will be further investigated. This will include further study of congenic mice on an NZB background, MRL/1 background, BXSB background or NZB/NZW background, but with B-cell hypoactivity, as well as recombinant inbred lines. The availability of athymic nude mice will allow us to study lack of thymic function in different strains with and without super-infection with different viruses or immunization with nucleic acid antigens. We hope thereby to define the relative contributions of genetic and different immunologic factors in the development of autoimmunity. The role of anti-idiotypic antibodies in control of autoantibody production will be investigated further.

Significance to Bio-medical Research and the Program of the Institute:

1. The genetic basis for autoimmunity is becoming much better established. The studies of B-cell defective mice point to potential genetic manipulations.

2. Studies of NZB mice indicate that there are both T cell abnormalities and B cell abnormalities which are separate and independent of each other.

3. Anti-DNA is produced by largely non-overlapping B cell subsets in NZB and MRL/1 mice.

4. Study of New Zealand and MRL/1 mice indicates that there are different cellular bases for SLE. This may lead to different treatment for different patients in the future.

Publications:

1. Raveche, E.S., Tjio, J.H., and Steinberg, A.D.: Genetic studies in NZB mice. II. Hyperdiploidy in the spleen of NZB mice and their hybrids. Cytogenetics Cell Genetics 23:182-193, 1979.

2. Huston, D.P., Rich, R.R., Steinberg, A.D., and Truitt, G.A.: Regulation of cytotoxic lymphocyte response in New Zealand mice by allo-antigen-activated spleen cells. J. Immunol. 122:1476-1481, 1979.

3. Raveche, E.S., Tjio, J.H., and Steinberg, A.D.: Genetic studies in NZB mice. III. Induced antinucleic acid antibody production. J. Immunol. 122:1454-1460, 1979.

4. Raveche, E.S., Tjio, J.H., and Steinberg, A.D.: Genetic studies in NZB mice. II. Hyperdiploidy in the spleen of NZB mice and their hybrids. Cytogenetics Cell Genetics 23:182-193, 1979.

5. Taurog, J.D., Moutsopoulos, H.M., Rosenberg, Y.J., Chused, T.M., and Steinberg, A.D.: The CBA/N X-linked defect prevents NZB B-cell hyperactivity in F_1 mice. J. Exp. Med. 150:31-43, 1979.

6. Attallah, A.M., Steinberg, A.D., Ahmed, A., and Sell, K.W.: Loss of lymphocyte chalone in mice with autoimmune disease. Int. Arch. Allergy Applied Immunol. 59:186-191, 1979.

7. Raveche, E.S., Tjio, J.H., Steinberg, A.D.: Genetic studies in NZB mice. IV. The effect of sex hormones on the spontaneous production of anti-T cell autoantibodies. Arthritis Rheum. 23:48-56, 1980.

8. Huston, D.P., Raveche, E.S., Steinberg, A.D.: Preferential lysis of undifferentiated thymocytes by natural thymocytotoxic antibodies from New Zealand Black mice. J. Immunol. 124:1635-1641, 1980.

9. Taurog, J.D., Smathers, P.A., Steinberg, A.D.: Evidence for abnormalities in separate lymphocyte populations in NZB mice. J. Immunol. (in press).

10. Steinberg, A.D., Roths, J.B., Murphy, E.D., Steinberg, R.T., Raveche, E.S.: Effects of thymectomy or androgen administration upon the autoimmune disease of MRL/Mp-lpr/lpr mice. J. Immunol. (in press).

11. Reinertsen, J.L., Kaslow, R.A., Klippel, J.H., Hurvitz, A.I., Lewis, R.M., Rothfield, N.F., Zvaifler, N.J., Steinberg, A.D., Decker, J.L.: An epidemiologic study of households exposed to canine systemic lupus erythematosus. Arthritis Rheum. 23:564-568, 1980.

12. Huston, D.P., Steinberg, A.D.: Kinetic analysis of NZB cytotoxic lymphocyte responses. J. Exp. Med. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41021-12 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Antinuclear antibodies in spontaneous and drug-induced SLE and other diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D. Steinberg	Senior Investigator	ARB NIAMDD
OTHER:	J.S. Cowdery	Clinical Associate	ARB NIAMDD
	J.D. Taurog	Clinical Associate	ARB NIAMDD
	E.S. Raveche	Chemist	LEP NIAMDD
	J.P. Reeves	Chemist	ARB NIAMDD
	K. Melez	Guest Worker	PMB NICHD
	P.A. Smathers	Biologist	ARB NIAMDD

COOPERATING UNITS (if any)
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LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies to nucleic acids are important diagnostically and pathogenetically in systemic lupus. The production of these antibodies is in part controlled by the X-chromosome through regulation of maturation of B cells destined to make IgM antibodies to nucleic acids. Sex chromosomes indirectly influence the magnitude to auto-antibody production through sex hormones: male sex hormones suppress the production of anti-nucleic acid antibodies. Antibodies to transfer RNA are produced by patients with SLE; these cross-react with viral nucleic acids. Subpopulations of patients have specific auto-antibodies.

Hybridoma cells producing anti-nucleic antibodies (derived originally by fusing BALB/c and NZB x NZW F_1 cells) preferentially grow and preferentially produce different autoantibodies in different recipient strains. These studies suggest that control mechanisms exist for the proliferation of, and perhaps synthesis by, B cells genetically destined to produce antibodies to nucleic acids.

Project Description:

Objectives:

Study the characteristics of a variety of antinuclear antibodies in human and murine "autoimmune diseases." Determine genetic contributions to the immune response to nucleic acid and related antigens.

Methods Employed:

Farr ammonium sulfate precipitation assay using radiolabeled ligands. Standard precipitin reaction using labeled and unlabeled nucleic acids or nuclear extracts. Hemagglutination, complement fixation and immunodiffusion assays. Standard immunization procedures.

Major Findings:

1. Genetic studies of NZB x DBA F_1 and backcross mice indicate that a single gene locus (expressing itself in a gene dosage fashion) plays the major role in controlling production of anti-DNA.

2. Analysis of 22 recombinant inbred lines points to a single gene independent of the gene for anti-T cell antibodies. This trait was found in half of the lines.

3. Castration of male NZB x DBA or DBA x NZB mice led to accelerated anti-DNA production, both following immunization and spontaneously. This was suppressed by testosterone and further accelerated by estrodiol. Castration of 3 week old NZB/NZW males accelerates anti-DNA production; castration at 3 months does not. Testosterone treatment of 3 week NZB/NZW females retards anti-DNA production; treatment at 3 months does not.

4. NZO mice develop both anti-DNA antibodies and diabetes.

5. Hybridomas produced by fusion of BALB/c-derived myeloma cells (non-producers) with NZB x NZW F_1 spleen cells produce antibodies to nucleic acids *in vitro* and when transferred to NZB x NZW F_1 recipients. Such hybridomas show preferential growth in different strains and preferential production of anti-nucleic acid antibodies with certain specificities.

Significance to Bio-Medical Research and the Program of the Institute:

Male sex hormones appear to suppress auto-antibody production explaining the more severe disease and greater incidence in females. This effect occurs primarily very early in life; it suggests that hormones modulate a maturational event in the immune system which in turn regulates autoimmune potential. Genes for high and low responsiveness to DNA may be one of the genetic contributions of autoimmunity in SLE.

Proposed Course:

Further define the genetics of the antibody response to DNA and try to correlate with other immune abnormalities. Further study the characteristics of

of antinuclear antibodies. Continue to evaluate the role of sex hormones in autoimmunity. Compare antinuclear antibodies of patients with different autoimmune diseases. Further study subpopulations of patients with a high incidence of autoimmunity.

Publications:

1. Huston DP, Steinberg, AD: Animal models of human systemic lupus erythematosus. Yale J. Biology and Medicine 52:289-305, 1979.

2. Raveche ES, Tjio JH, Boegel WA, Steinberg AD: Studies of the effects of sex hormones on autosomal and X-linked genetic control of induced and spontaneous antibody production. Arthritis Rheum. 22:1177-1187, 1979.

3. Taurog JD, Smathers PA, Steinberg AD: Evidence for abnormalities in separate lymphocyte populations in NZB mice. J. Immunol. (in press).

4. Steinberg AD, Roths JB, Murphy ED, Steinberg RT, Raveche ES: Effects of thymectomy or androgen administration upon the autoimmune disease of MRL/Mp-lpr/lpr mice. J. Immunol. (in press).

5. Melez KA, Harrison LC, Gilliam JN, Steinberg AD: Autoimmunity in the NZO (New Zealand Obese) mouse. Diabetes (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41022-09 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Therapeutic Studies in New Zealand Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D. Steinberg	Senior Investigator	ARB NIAMDD
	J.P. Reeves	Chemist	ARB NIAMDD
	K. Melez	Guest Worker	PMB NICHD
	P. Smathers	Biologist	ARB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIH, NIAMDD, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Non-specific immunosuppressive therapy of man is associated with considerable morbidity. Some regimens may be superior to others. The New Zealand mice offer a convenient model for studying different drug regimens prior to study in patients. Soluble suppressor substances have prolonged life in NZB/NZW mice.

Treatment of NZB/NZW mice with Ribavirin, a broad spectrum antiviral drug, has been very effective. Renal deposits of immune complexes can be prevented. Such treatment is potentially applicable to humans with SLE.

Male sex hormones, testosterone and 5 alpha-dihydrotestosterone (but not Danazol) prolonged life in female NZB/NZW mice. Such treatment was effective even when started after clinical illness and did not require castration for its full effect. However, NZB mice are much less sensitive to androgen administration.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

NZB/NZW F_1 mice spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. NZB mice develop Coomb's positive hemolytic anemia, membranous nephrosis and excessive lymphoreticular proliferation. These mice are excellent models for comparing different immunosuppressive and anti-viral drug programs. In addition, as the immunologic basis for the disease becomes better understood, they offer a model for studying more specific immunotherapy.

Methods Employed:

1. Comparison of different immunosuppressive and anti-viral drug regimens in natural history of NZB/NZW mice by study of antibodies to DNA, proteinuria and survival.

2. Treatment with suppressor cells and/or suppressor substances derived from such cells in an attempt at restoring normal regulatory functions and improve the natural history of New Zealand mice.

3. Treatment of NZB/NZW and NZB mice with androgens.

Major Findings:

1. Supernatants from BALB/c spleen cells stimulated with Concanavalin A were given to NZB/NZW mice starting at 18-19 weeks of age. Such mice had prolonged survival as compared with mice receiving inactive control supernatants. The 10,000 molecular weight fraction was at least as effective as the whole supernatant.

2. Androgens prolong life in female NZB/NZW mice when treatment is started before and when started after the onset of illness. Both testosterone and dihydrotestosterone were therapeutically effective in a dose-dependent manner. Danazol was of no benefit.

3. NZB mice had a transient reduction in anti-RBC and anti-DNA antibodies with androgen treatment, but no increase in survival.

4. MRL/Mp-lpr/lpr mice had a decrease in anti-DNA proteinuria and an increase in survival with androgen treatment. Their lymphadenopathy was only slightly reduced with androgens.

Significance to Bio-Medical Research and the Program of the Institute:

Possible sex hormonal intervention in SLE should be viewed with reduced enthusiasm since NZB mice showed so little benefit. Continued study in mice may provide the background information necessary for immunological intervention in humans with SLE. Suppressor factors may be of value in humans in the future.

Proposed Course:

Further study of sex hormones in other SLE strains of mice, expecially MRL and F_1 crosses of BXSB mice with other strains to determine whether or not such treatment is uniformly effective.

Publications:

1. Steinberg, A.D., and Decker, J.L.: Immunoregulatory Drugs. In McCarty, D.J. (Ed.): Arthritis and Allied Conditions. Philadelphia, Lea & Febiger, 1979, pp. 375-390.

2. Steinberg, A.D., Melez, K.A., Raveche, E.S., Boegel, W.A., Smathers, P. A., Taurog, J.D., Weinlein, L.M., and Duvic, M.: Approach to the study of the role of sex hormones in autoimmunity. Arthritis Rheum. 22:1170-1176, 1979.

3. Melez, K.A., Boegel, W.A., Steinberg, A.D.: Therapeutic studies in New Zealand Mice. VII. Successful treatment of NZB/NZW F_1 females of different ages with androgens. Arthritis Rheum. 23:41-47, 1980.

4. Steinberg, A.D., Roths, J.B., Murphy, E.D., Steinberg, R.T., Raveche, E.S.: Effects of thymectomy of androgen administration upon the autoimmune disease in MRL/Mp-*lpr*/*lpr* mice. J. Immunol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41023-06 ARB

PERIOD COVERED
October 1, 1979 to September 31, 1980

TITLE OF PROJECT (80 characters or less)
Study of Immune Abnormalities in Patients with Systemic Lupus Erythematosus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	R.M. Blaese	Senior Investigator	IB NCI
	J. Grayson	Clinical Associate	IB NIC
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COOPERATING UNITS (if any)
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Laboratory of Clinical Investigations, NIAID

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Excessive B cell activity and loss of regulatory cells occurs in patients with active SLE; these abnormalities are less marked when the disease is quiescent. Nevertheless, excessive B cell proliferation occurs even in inactive patients.

Loss of a subpopulation of regulatory T cells occurs in patients with active SLE. Antibodies to the precursors of suppressor T cells are spontaneously produced by the patients. Such antibodies prevent normal T cell control of abnormal B cell activity allowing perpetuation of autoantibody production. Some patients produce antibodies to a subset of T cells which regulates T cells but not B cell immunity. Such patients are less ill than those which produce antibodies capable of interfering with regulation of B cell function. The same patients which have impaired suppressor T cell function when active regain normal suppressor T cell function when the disease is quiescent. Studies with monoclonal anti-T cell antibodies indicate that during active disease there is a quantitative decrease in suppressor cells which is not present when the disease is inactive.

Project Description:

Objectives:

To apply the newer understanding of basic immunologic principles, and observations in mice who develop lupus-like diseases, to the study of patients with SLE.

Methods Employed:

1. Study of families of patients with SLE or larger groups of patients with a high incidence of SLE. A battery of immunologic measurements would be made in such patients.

2. Study of lymphocytes and antibodies to lymphocytes from patients with SLE.

   a. Number and distribution of T, B and null cells and their responses to mitogens or allogeneic cells.

   b. Study of antibody producing plaque forming cells (PFC) in the peripheral blood of patients.

   c. Study of generation of suppressor cells by activating purified T cells with concanavalin A and adding them to a second culture.

   d. Study of other T cell-B cell interactions with the autologous MLR.

   e. Relationship between anti-lymphocyte antibodies in patients with SLE and abnormalities of cell number and/or function.

   f. Study of anti-lymphocyte antibodies by use of the fluorescence activated cell sorter (FACS). Additional analyses use monoclonal anti-T cell antibodies.

   g. Study of anti-lymphocyte antibodies by complement-mediated lysis, dye exclusion, and antibody dependent direct cellular cytotoxicity (ADCC).

Major Findings:

1. Anti-lymphocyte antibodies occur in association with increased activity of patients with SLE. These antibodies can reduce the number of peripheral T cells and interfere with their function, especially suppressor cell generation.

2. Antibodies to T cells present in the plasma of patients with active systemic lupus erythematosus (SLE) plus complement are able to eliminate concanavalin A-induced suppressor function for the proliferative responses of T cells to allogeneic lymphocytes (MLR) and of B cells to pokeweed mitogen (PWM). Such antibodies were found to be effective in eliminating suppressor function only when T cells were treated prior to activation; there was no effect when treatment was performed after activation. These studies indicate that the antibodies preferentially interact with a T cell necessary for the generation of suppressor cells, rather than with mature, activated suppressor cells. Studies of individual SLE patients

indicated that the same defects observed in SLE T cells were induced in normal T cells by plasma from that patient. Such observations suggest that some T cell defects associated with active SLE may not be intrinsic T cell abnormalities, but, rather, secondary effects of anti-T cell antibodies.

3. Patients with active SLE have increased quantities of antibody producing PFC to defined chemical haptens. Increases in IgG antibody producing cells especially characterized patients with active SLE.

4. Using the reverse plaque assay, active SLE was found to correlate with IgG antibody producing cells better than with anti-DNA, C3, and other typical serologic measures. Of interest, serum IgG concentration correlated very well with active SLE.

5. Serial study of patients with SLE indicate that defects in autologous MLR and in suppressor cell function occur during active, but not inactive, disease in each patient.

6. IgG anti-T cell antibodies in SLE plasma eliminate T cells by antibody dependent cellular cytotoxicity (ADCC) at 37°C. This is a possible mechanism of in vivo removal of T cells.

7. Studies with monoclonal antibodies, which identify T cell subsets, indicate that patients with active SLE have a marked decrease in suppressor cells and suppressor cell precursors (OKT5 and OKT8 positive cells). Such cells are present in normal numbers in patients whose disease has become inactive.

Significance to Bio-Medical Research and the Program of the Institute:

Populations for further study are being defined and techniques refined for continued study of humans with SLE. It is hoped that the rewarding observations in the animal models of SLE can continue to be applied to the study of the human disease itself.

The studies to date suggest that patients with SLE have generalized B cell hyperactivity rather than selective B cell hyperactivity towards self-antigens. However, both genetic factors and environemntal exposure determine which B cell clones will be expanded. Loss of suppressor and helper cells in human SLE is associated with (a) disease activity, and (b) anti-T-cell antibodies. These defects preferentially occur during disease activity, indicating that the abnormalities are not permanent, and that appropriate specific prevention may be possible. The T cell dysfunction may be, in part, secondary to anti-T cell antibody production. This will direct primary therapeutic attention towards the excessive B cell activity and away from defective T regulatory function.

Proposed Course:

Continue the studies initiated.

Publications:

1. Pons, V.G., Reinertsen, J.L., Steinberg, A.D., and Dolin, R.: Decreased cell mediated cytotoxicity against virus-infected cells in systemic lupus erythematosus. J. Med. Virology (in press). 1980.

2. Sakane, T., Steinberg, A.D., Arnet, F.C., Reinertsen, J.L., and Green, I.: Studies of immune functions of patients with systemic lupus erythematosus. III. Characterization of the lymphocyte subpopulations responsible for the defective autologous MLR. Arthritis Rheum. 22:770-776, 1979.

3. Sakane, T., Reeves, J.P., Steinberg, A.D., and Green, I.: Studies of immune functions of patients with systemic lupus erythematosus. Complement dependent immunoglobulin M anti-thymus-derived cell antibodies preferentially inactive suppressor cells. J. Clin. Invest. 63:954-965, 1979.

4. Raveche, E.S., and Steinberg, A.D.: Lymphocytes and lymphocyte functions in SLE. Clinics in Haematology 16:344-370. 1979.

5. Sakane, T., Steinberg, A.D., Reeves, J.P., Green, I.: Studies of immune functions of patients with systemic lupus erythematosus. T cell subsets and antibodies to T cell subsets. J. Clin. Invest. 64:1260-1269, 1979.

6. Steinberg, A.D.: Studies of immune regulation in patients with systemic lupus erythematosus. In Decker, J.L. (Moderator), Systemic lupus erythematosus: Evolving concepts. Ann. Intern. Med. 91:587-592, 1979.

7. Klippel, J.H., Karsh, J., Stahl, N.I., Decker, J.L., Steinberg, A.D., Schiffman, G.: A controlled study of pneumococcal polysaccaharide vaccine in systemic lupus erythematosus. Arthritis Rheum. 22:1321-1325, 1979.

8. Steinberg, A.D., Sakane, T., Glinski, W., Klassen, L.W., Green, I., Merchant, E.B., Budman, D.R., Reeves, J.P., Becker, T.M.: Abnormalities of immune regulation in systemic lupus erythematosus. In Kilburn, D.G., Levy, J.G., and Teh, H.S. (Eds.): Regulation of T Cells. Vancouver, B.C., University of British Columbia, 1969, pp. 31-76.

9. Sakane, T., Steinberg, A.D., Green, I.: Studies of immune functions of patients with systemic lupus erythematosus. V. T-cell suppressor function and autologous MLR during active and inactive phases of disease. Arthritis Rheum. 23:225-231, 1980.

10. Moutsopoulos, H.M., Klippel, J.H., Pavlidis, N., Wolf, R.O., Sweet, J.B., Steinberg, A.D., Chus, F.C., Tarpley, T.M.: Correlative histologic and serologic findings of sicca syndrome in patients with systemic lupus erythematosus. Arthritis Rheum. 23:36-40, 1980.

11. Steinberg, A.D., Sakane, T., Klassen, L., Green, I, Williams, G.W., Budman, D.: Naturally occurring anti-T cell antibodies in patients with SLE interfere with normal immune regulatory function. In Homma, M., and Fukase, M., (Eds.): Proc. International Meeting on SLE, Kyoto, Japan, Nov. 1978. Tokyo University Press, In press.

12. Karsh, J., Halbert, S.P., Klima, E., Steinberg, A.D.: Quantitative determination of rheumatoid factor by an enzyme labeled immunoassay. J. Immunol. Methods 32:115-126, 1980.

13. Herscowitz, H.B., Sakane, T., Steinberg, A.D., Green, I.: Heterogeneity of human suppressor cells induced by Conconavalin A as determined in simultaneous assays of immune function. J. Immunol. 124:1403-1410, 1980.

14. Reinertsen, J.L., Kaslow, R.A., Klippel, J.H., Hurvitz, A.I., Lewis, R.M., Rothfield, N.F., Zvaifler, N.J., Steinberg, A.D., Decker, J.L.: An epidemiologic study of households exposed to canine systemic lupus erythematosus. Arthritis Rheum. 23:564-568, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-41025-09 ARB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on the cell surface receptor for IgE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Metzger	Chief, Sec. Chem. Immunol.	ARB/NIAMDD
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	C. Fewtrell	Guest Worker	ARB/NIAMDD
	D. Holowka	Guest Worker	ARB/NIAMDD
	A. Goetze	Guest Worker	ARB/NIAMDD
	B. Rivnay	Visiting Associate	ARB/NIAMDD
	E. Kempner	Senior Investigator	LPB/NIAMDD

COOPERATING UNITS (if any) Div. Bacterial Prod., Bureau Biol. (T.Y. Liu)
Dept. Memb. Res., Weizman Inst. of Sci., Israel (C. Gitler, T. Beroovici)
Department of Medicine, Johns Hopkins University (A. Sobotka and L. Lichtenstein)

LAB/BRANCH Arthritis and Rheumatism Branch

SECTION Chemical Immunology

INSTITUTE AND LOCATION NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7	6	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are pursuing an in-depth investigation on the structure and function of the membrane receptor for IgE. For these studies intact cells from a rat basophilic leukemia as well as normal rat mast cells are being used. In addition receptor-bearing membrane vesicles and solubilized receptors are being studied. The aim is to understand the structure of the receptor as well as its integration in the plasma membrane. Results of such studies as well as direct functional studies are aimed at understanding the early stimulation of cells by antigen-induced, antibody-mediated reactions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

IgE is a unique class of immunoglobulin which plays a predominant role in the physiological events associated with a variety of allergic phenomena. The immunoglobulin becomes attached to the surface of basophils and mast cells and, upon exposure to the allergenic antigen, mediates the release of histamine and other vasoactive amines from the cells to which it is attached. Although there is an increasing amount of information concerning the biochemistry of this reaction almost nothing is known about how the IgE acts as the cell surface transducer. The present project is directed towards increasing our understanding of how the IgE interacts with mast cells. In addition we wish to understand the mechanism by which the ternary complex of antigen-IgE antibody-cell surface receptor triggers the degranulation of basophilic cells.

Methods Employed:

Three important new approaches have been employed:

a) Target analysis (see sec. B1C)

b) Use of hydrophobic photolyzable modifying reagents (see sec. B2b).

c) Use of artificial liposomes (see Propose course - 3).

Major Findings:

Since our previous report the following has been found:

A. Functional Studies:

1. Nature of triggering unit

Work on the use of defined oligomers of IgE to determine the type of receptor aggregates which lead to optimal stimulation of mast cells and their tumor analogs has been completed (C. Fewtrell and H. Metzger J. Immunol. In press). Mathematical analysis of the results with the tumor cells indicated that the differences between dimers and trimers was not merely quantitative in the sense that trimers do not simply yield an increased number of effective dimers. Rather, the data suggest that trimers and higher oligomers yield qualitatively different signals suggesting that the triggering mechanism is one which distinguishes between oligomers of receptors. Of interest, secretion from normal rat mast cells shows only minimal differences in the response to the various IgE oligomers. We have postulated that normal mast cells also have a mechanism which distinguishes between various oligomers but that they are sufficiently responsive to dimers so that the size of the oligomer does not importantly affect the response.

Work with colleagues at Johns Hopkins in which the response of human basophils to human IgE are being similarly studied is almost complete also. The results with the basophils suggest smaller differences in the effectiveness of the various oligomers in stimulating secretion. However, under sub-optimal conditions of incubation, the higher oligomers again show substantially greater activity than the dimers.

2. Work on Ca^{2+} fluxes is continuing, although no major new findings have been made. Ca^{2+} influx studies have been disappointing because high backgrounds and considerable scatter make detailed analysis of the small amount of enhanced uptake difficult. The future course of this work has not been resolved.

3. Work on ionophore stimulated release is complete and a manuscript is in preparation.

4. Over the past year a variety of studies have been performed which have examined the response of the tumor cells to a variety of reagents used in the studies of others on secretion from normal mast cells and basophils. A summary of the results is In Press. We have concluded that the basic mechanism of IgE-mediated triggering in the tumor cells is similar to that observed with normal cells.

B. Structure Studies

The major new finding is that the receptor for IgE appears to be composed of two polypeptide chains rather than only one as heretofore thought. This is based principally on the finding that a second polypeptide which had previously been found to become cross-linked to the polypeptide which binds IgE, has the properties of a subunit under conditions where the receptor is isolated under very mild conditions. The chains have been dubbed α and β. Table I indicates some of the basic properties of the chains.

Table I

Subunit Structure of Receptor for IgE

Property	α	β
Size	50K*	30K*
Composition		
carbohydrate	~15K*	none*
polypeptide	~35K*	30K*
Domains	~ 30K + ? other *	20K + ? other *
Exposure		
surface	+ (30K domain) *	not detected *
intra membranous	not detected	+ (20K domain)*
cytosol	?	?
Interactions	with β -chain via ? domain	with α -chain via 20K domain*
Activity	binds IgE (30K domain)*	does not affect binding of IgE by α *

*New findings

1. α -Chain

   a. The compositional analysis has been completed (J. Kanellopoulos, T.Y. Liu, G. Poy, H. Metzger. J. Biol. Chem. In press). By applying a known number of mols to the analyzer the compositional analysis could be used to determine the contributions of the polypeptide and carbohydrate portions to the molecular weight. Over 30% of the molecular weight is due to carbohydrate. The 'hydrophobic index' shows a value between that observed for peripheral and integral membrane proteins.

   b. When subjected to a variety of proteases in sodium dodecyl sulfate, the α chain which has been derived from surface-labeled receptors yields principally one component with an apparent molecular weight of 32K. Subsequent digestion yields two smaller products having apparent molecular weights of 20 and 12K. Interestingly purified isolated α chains when iodinated show the same pattern. Thus a portion of the α -chain appears to be in a rather stable 30K domain which by evidence from differential labeling studies is the site of binding of IgE.

   c. In target analysis the size of a functional unit is assessed by assaying its activity after exposure to ionizing radiation. We have begun studies in which the size of the unit which is critical for binding IgE is determined. If this function were

dependent on the presence of β -chain or another still undetected chain then such an analysis would be expected to show a target size larger than the α -chain. In studies on solubilized receptors we have found a molecular weight that actually is substantially lower than the α -chain. Interestingly the size of the target is similar to the 30K domain of the α -chain referred to above. Such a result is to our knowledge unprecedented and suggests that contrary to previous findings, a functional unit by this type of analysis may be smaller than the smallest covalent structure which carries it. Further analyses are in process.

2. β-chain

a. Previous studies had shown that the polypeptide we now call the α-chain became crosslinked to a cellular component when the crosslinking is performed on intact cells or in detergent extracts. This material could not be surface labeled; we could only detect it with intrinsic labeling by ^{3}H-amino acids. In order to analyze the material receptor preparations required extensive, albeit apparently mild purification procedures. These conditions apparently lead to α , β dissociation so that β is reproducibly recovered only when cross-linked to . We have now found that with more rapid or no purification (i.e. using immunoprecipitation only) the β -chain is regularly associated with the α -chain. These studies became feasible when we determined that the β -chain becomes modified by iodonapthylazide.

b. Iodonaphthylazide (INA) is a reagent devised by Prof. C. Gitler. It is very apolar, can be prepared at high specific activity and upon photolysis at 310 nm forms a nitrene which can insert into a variety of covalent bonds. We have reacted this reagent with intact cells, and membrane preparations of the cells. We have found that only the β -chain becomes labeled with no detectable modification of the α -chain.

c. Preliminary data suggest that under acidic conditions an endogenous protease cleaves the β -chain into a 20K fragment and as-yet-undefined other breakdown material. The 20K fragment can be cross-linked to the α -chain and is labeled with INA. We postulate that the 20K 'domain' is at least partially intramembranous and carries that portion of the β -chain with which the α-chain interacts.

Significance to Bio-Medical Research and the Program of the Institute:

The IgE Mast cell system is of significance in the pathophysiology of allergy, parasitic infections, and inflammation. It is also an experimentally convenient system to study mechanisms of antigen-induced antibody-mediated cellular reactions in general. Finally it is an excellent model for the study of exocytosis. So far we are continuing to make good progress in the elucidation of the molecular mechanisms of this system. Knowledge of

these may eventually permit rational intervention in situations where the system contributes to pathology.

Proposed course:

1. Continuation of structural studies on α-chain. In particular continue working with proteolytic cleavage products, use differential labeling reagents, analyze fragments for carbohydrate and polypeptide content and, if they can be prepared preparatively, on compositional analyses. If appropriate start 10 sequence work on 1 or more fragments. Complete target analysis.

2. Begin detailed analysis of β-chain and how it interacts with α-chain.

3. Reconstitution studies. Initial studies are underway in which we are attempting to incorporate IgE-receptor complexes into liposomes. If successful such liposomes can be used to study:

   a. the integration of the receptor into lipid bilayers.

   b. the ability of such liposomes to reincorporate receptor in a functional form into cells.

   c. to study possible changes in the receptor upon aggregation.

4. Initiate search for changes in the receptor as a consequence of triggering intact cells.

Publications

1. Kanellopoulos, J. Rossi, G. and Metzger, H. : Preparative isolation of the membrane receptor for immunoglobulin E. J. Biol. Chem. 254: 7691-7697, 1979. (previously listed as "In Press")

2. Metzger, H. : Antibodies as cell surface receptors. In C. DeLisi & R. Blumenthal (Eds.): Physical chemical aspects of cell surface events in cellular recognition. Elsevier N. Holland, New York, 1979 pp 237-241.

3. Metzger, H. : IgE role in mast cell exocytosis. INFO 12: 1-4, 1979/

4. Kanellopoulos, J., Rossi, G. and Metzger, H. : Preparative isolation of the cell receptor for immunoglobulin E. J. Biol. Chem. 254: 7691-7697, 1979.

5. Fewtrell, C., Geier, M., Goetze, A., Holowka, D., Isenman, D.E., Jones, J.F., Metzger, H., Navia, M., Sieckmann, D., Silverton, E. and Stein, K. : Mediation of effector functions by antibodies: Report of a workshop. Molec. Immunol. 16: 741-754, 1979.

6. Holowka, D., Hartmann, H., Kanellopoulos, J. and Metzger, H. : Association of the receptor for immunoglobulin E with an endogenous polypeptide on rat basophilic leukemia cells. J. Receptor Res. (In press) 1980.

7. Fewtrell, C. and Metzger, H. : Larger oligomers of IgE are more effective than dimers in stimulating rat basophilic leukemia cells. J. Immunol. (In press) 1980.

8. Fewtrell, C. and Metzger, H. : The role of aggregation in the function of the IgE Fc receptors. J. Retic. Endo. Soc. (In press) 1980.

9. Kanellopoulos, J., Liu, T.Y., Poy, G., and Metzger, H. : Composition and subunit structure of the cell receptor for immunoglobulin E. J. Biol. Chem. (In press) 1980.

10. Metzger, H., Kanellopoulos, J., Holowka, D., Goetze, A. and Fewtrell, C. : Structural analyses of the receptor for IgE on rat basophilic leukemia cells. Biochem. of Acute Allergic Reactions. (In press) 1980.

11. Fewtrell, C. and Metzger, H. : Stimulus-secretion coupling in rat basophilic leukemia cells. Biochem. of Acute Allergic Reactions (In press) 1980.

12. Dessaint, J-P, Waksman, B.H., Metzger, H. and Capron, A. : Cytophilic Binding of IgE to the macrophage. III. Involvement of cyclic GMP and calcium in macrophage activation by dimeric or aggregated rat myeloma IgE. Cellular Immunol. 51: 280-292, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 41028-05 ARB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Renal effects of aspirin and other non-steroidal anti-inflammatory drugs in SLE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PROJECT TERMINATED

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41029-04 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Development of Computerized Clinical Database for Systemic Lupus Erythematosus (SLE)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Klippel	Senior Investigator	ARB NIAMDD
OTHER:	J.L. Decker	Chief	ARB NIAMDD
	E.K. Harris	Chief	LAS DCRT
	G. Shakarji		DMB DCRT

COOPERATING UNITS (if any)
Laboratory of Applied Studies, DCRT
Data Management Branch, DCRT
Laboratory of Statistical and Mathematical Methodology, DCRT

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The evaluation of long term randomized therapeutic studies of SLE would be facilitated by the development of a comprehensive clinical database. In this project, an ongoing computerized storage and retrieval system will be used to monitor clinical and therapeutic information on SLE patients participating in studies of immunosuppression and diffuse glomerulonephritis.

Project Description:

Objectives:

Long term randomized trials comparing the efficacies of several different forms of immunosuppressive therapies and prednisone in the treatment of SLE diffuse glomerulonephritis have been in progress within our branch since 1970. These studies require careful monitoring of sequential observations of drug administration, clinical and laboratory studies and complications to determine the superiority of any single program. It is the objective of this project to develop a computerized data storage and retrieval system to monitor and evaluate these clinical trials.

Methods Employed:

A time oriented database will be used to record drug administration, clinical manifestations, studies of renal function, relevant laboratory studies and select complications of SLE patients participating in immunosuppressive studies. Information will be made current at the time of each inpatient, as well as outpatient, Clinical Center visits. The entry of data will be made from terminals located within the Clinical Center and datafile stored at DCRT.

Major Findings:

None at this time.

Significance to Bio-Medical Research and the Program of the Institute:

This project will benefit ARB clinical research activities by (1) facilitating the monitoring and assessment of therapeutic studies of SLE, (2) permitting analysis of interrelationships of multiple variables in the course of SLE and (3) improvement of the care of SLE patients participating in ARB studies.

Proposed Course:

The entry of data will be made both from retrospective information collected from patients involved in current trials and prospectively once a file has been established. Analyses of changes in renal function using a commulative summation technique will be used to compare the relative efficacies of different immunosuppressive therapies.

Publications:

None at this time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41030-04 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Clearance of immune complexes by patients with systemic lupus erythematosus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PROJECT TERMINATED

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41035-03 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Lymphocyte Depletion in the Treatment of Rheumatoid Arthritis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.L. Decker	Chief	ARB NIAMDD
	J. Karsh	Guest Worker	CIB NIDR
OTHER:	J.H. Klippel	Senior Investigator	ARB NIAMDD
	A.B. Deisseroth	Chief	POB NCI
	D.G. Wright	Senior Investigator	POB NCI
	W. Flye	Senior Investigator	SURG NCI

COOPERATING UNITS (if any)

Experimental Hematology Section, DCT NCI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project will evaluate the effect of removal of large numbers of peripheral blood mononuclear cells in patients with severe rheumatoid arthritis. Vascular access will be obtained by forearm arteriovenous fistula and lymphapheresis performed three times weekly for 10 weeks using a continuous flow cell separator. The removed cells will be studied for membrane characteristics and in vitro assays of immune function. The clinical activity of the arthritis will be quantitated by a global activity index during and following completion of the project.

Project Description:

Objectives:

Although the etiology of RA is not known, involvement of both the cellular and humoral immune systems are thought to play a basic role in the pathogenesis of this chronic systemic disorder. This is best evidenced by the favorable effect on RA of cytotoxic, or immunosuppressive, drugs and lymphocyte depletion by prolonged drainage of the thoracic duct. This project will evaluate the effect on RA of the removal of peripheral blood mononuclear cells using a continuous flow cell separator.

Methods Employed:

Patients with severe active RA unresponsive to conventional therapies will participate by informed consent. A temporary forearm side to side arteriovenous fistula will be constructed for purposes of obtaining repeated vascular access. Three times weekly lymphocyte removal using an Aminco continuous flow cell separator will be performed for a period not to exceed 10 weeks. The activity of the RA will be quantitated weekly using a standard global activity index. The frequency of lymphapheresis will be altered according to degrees of responsiveness. The removed cells will be quantitated and studied for lymphocyte subpopulations, surface membrane characteristics and in vitro immune function.

Major Findings:

Four patients with seropositive, erosive RA have been studied. In the course of 14 to 18 lymphapheresis procedures per patient, a mean of 11.1×10^{10} by lymphocytes and 2.1×10^{10} monocytes have been removed. This has resulted in a greater than 50% improvement (mean 68%) of the Articular Index in all patients and persisted for up to 19 weeks after discontinuation of lymphapheresis.

Significance to Bio-Medical Research and the Program of the Institute:

The removal of lymphocytes from the circulation should lead to an early diversion of cells from the inflammed synovium and an eventual depletion of select cell populations. The rate of reduction of synovial inflammation during lymphapheresis and its anticipated recurrence after discontinuing the procedure at 10 weeks will be observed to assess the potential therapeutic usefullness of this type of intervention.

The importance of circulating mononuclear cells, lymphocytes and monocytes, in chronic RA inflammation will be studied.

Proposed Course:

The project was completed and reported. It formed the basis for a sequential study.

Publications:

1. Karsh J, Wright DG, Klippel JH, Decker JL, Deisseroth AB, Flye MW: Lymphocyte depletion by continuous flow cell centrifugation in rheumatoid arthritis. Arthritis Rheum, 22:1055-1059, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41036-03 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Epidemiology of Canine and Human Systemic Lupus Erythematosus (SLE)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. H. Klippel	Senior Investigator	ARB NIAMDD
OTHER:	J.L. Decker	Chief	ARB NIAMDD
	A.D. Steinberg	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)
Arthritis and Immunologic Diseases Activity, Center for Disease Control, Atlanta, Georgia; St. Louis Park Medical Center, Minneapolis, Minnesota

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project will compare the incidence of clinical and serologic autoimmune abnormalities in members of households exposed to known cases of canine SLE or appropriately matched control dogs.

Project Description:

Objectives:

This project will evaluate the relationship between canine and human SLE, by means of systematic epidemiologic survey, comparing households exposed to known cases of canine SLE with appropriately matched control households.

Methods Employed:

1. Approximately 20 dogs with recently diagnosed SLE or high titered antinuclear antibodies will be identified with the cooperation and assistance of regional veterinary centers specializing in the laboratory evaluation and study of canine SLE. Appropriate control dogs will be selected and matched to the SLE dog for defined household characteristics.

2. The head of each household in which the dogs reside will be contacted and permission for study obtained.

3. Each "SLE dog" and "control dog" household will be visited and a systematic epidemiologic survey completed. This will include a chronologic record of persons present in the household during the tenure of the dog, an estimate of the intensity of dog-human contact for all household members exposed to the dog, and a thorough review of medical histories of household members.

4. Blood specimens will be obtained from all household members and evaluated for antinucleic acid antibodies, antinuclear antibodies, rheumatoid factors, immunoglobulins and lymphocytotoxic antibodies.

5. The incidence of clinical and laboratory abnormalities in SLE and control dog households will be analyzed according to chronologic and contact relationships with the dogs.

Major Findings:

The project has studied 83 members of 19 households who were exposed to a dog with high titer antinuclear antibodies and 50 members of 16 control households matched for dog age, sex and veterinarian. No differences between these two populations have been identified with regard to clinical SLE symptoms or antinuclear or lymphocytotoxic antibodies, rheumatoid factors or serum immunoglobulins. Thus, the study has not detected any clinical or serologic effect of human exposure to canine SLE.

Significance to Bio-Medical Research and Programs of the Institute:

There is evidence to suggest that both environmental and genetic factors are important in human SLE pathogenesis. This project will attempt to evaluate the important environmental consideration of the potential relationship between dog and human SLE.

Publications:

1. Reinertsen JL, Kaslow RA, Klippel JH, Hurvitz AI, Lewis RM, Rothfield NF, Zvaifler NJ, Steinberg AD, Decker, JL: An epidemiologic study of households exposed to canine systemic lupus erythematosus. Arthritis Rheum 23:564-568, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41037-03 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Genetic Factors in Systemic Lupus Erythematosus (SLE): Study of the Major Histocompatibility Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Klippel	Senior Investigator	ARB NIAMDD
	D.L. Mann	Senior Investigator	IB NCI
OTHER:	J.L. Decker	Chief	ARB NIAMDD
	A.D. Steinberg	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)
Immunology Branch, NCI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The multiple occurrences of SLE over several generations in families suggest the importance of genetic factors in SLE pathogenesis. In this project, alloantigens on B-lymphocytes, coded for by genes on the sixth chromosome known as the major histocompatibility complex, will be examined in patients with SLE. Select families with multiple occurrences of SLE will also be studied.

These alloantigens have physico-chemical and amino acid sequence homologies with murine B-cell antigens responsible for the regulation of specific immune responses. The frequency of individual alloantigens in SLE, as compared to normal controls, will be used in an attempt to identify immune genetic markers associated with SLE.

Project Description:

Objectives:

Specific cell surface antigens coded for by genes in the HLA-D region of the major histocompatibility complex have the potential to be associated with an increased susceptibility to autoimmune diseases. These human alloantigens can be detected on B-lymphocytes and monocytes and have physico-chemical and amino acid sequence homologies with murine B-cell antigens which regulate immune responses. These antigens can be identified by reactivity with B-lymphocyte alloantisera and, in certain instances, assignments of HLA-Dw specificities made on the basis of serologic reactivities.

In this project, we will test the hypothesis that specific HLA-D region genes are associated with SLE by determining B-lymphocyte alloantigens in lupus patients.

Methods Employed:

1. Lymphocytes from SLE patients will be obtained by Ficoll-Hypaque sedimentation and B-lymphocytes isolated by adherence to anti-human $F(ab)_2$ coated plastic plates.

2. B-lymphocytes will be typed by a complement-dependent microcytotoxicity assay using a serum panel from the Seventh International Histocompatibility Workshop (Oxford) and a second panel of 47 sera obtained from multiparous women.

3. HLA-DRw types will be assigned on the basis of reactivities of the Oxford Workshop panel.

4. The reaction frequencies of individual sera and HLA-DRw types will be compared between SLE patients and normal controls.

Major Findings:

1. B-lymphocyte alloantigens defined by the non-Workshop panel of 47 pregnancy sera have been compared in 53 patients with SLE and 184 normal controls. One serum, la-715, reacted with B-lymphocytes from 72% of SLE patients and 14% of controls (p_c 0.005, relative risk 15.4).

2. HLA-DRw assignments using the Workshop panel have been compared in 39 SLE patients and 490 controls. Both HLA-DRw2 (51% vs 26%, p_c 0.004) and HLA-DRw3 (49% vs 22%, p_c 0.004) were increased in systemic lupus erythematosus.

3. Lymphocyte typing studies of seven families with multiple occurrences of SLE have suggested that the coinheritance of several B-lymphocyte alloantigens may influence lupus susceptibility.

In summary, this study demonstrates that select B-lymphocyte alloantigens, which are controlled by genes in the major histocompatibility complex, are present in increased frequency in SLE.

Significance to Bio-Medical Research and Programs of the Institute:

Immune response genes have been extensively studied in a variety of species. These genes are located within a gene grouping called the major histocompatibility complex and code for cell surface antigens found predominantly on B-lymphocytes and macrophages. These antigens play a major role in the regulation of specific immune responses.

Human immune response genes have yet to be fully characterized. However, human immune responses appear to be influenced by genes linked to the major histocompatibility. Furthermore, human B-lymphocyte alloantigens have been identified which are structurally similar to murine B-cell antigens.

Immune response genes may influence an individual's susceptibility to autoimmune diseases. This hypothesis will be evaluated by determining B-cell alloantigens, which are coded for by genes present in the region of presumed immune response genes, in patients with SLE.

Publications:

1. Reinertsen JL: Immunogenetic aspects. In Decker JL, moderator. Systemic lupus erythematosus: evolving concepts. Ann Int Med 91:592-595, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41038-03 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Renal biopsy pathology in systemic lupus erythematosus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J.E. Balow — Senior Investigator — ARB/NIAMDD

COOPERATING UNITS (if any)
Armed Forces Institute of Pathology, Washington, D.C.
(T.A. Antonovych, M.D.)

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Detailed analysis of renal biopsy pathology has been undertaken in patients with systemic lupus erythematosus who are receiving various therapeutic regimens of immunosuppressive agents. Biopsies will be scored for various indices of pathological activity as well as for patterns of deposition of immune complexes by immunofluorescence and electron microscopy; these morphologic parameters are to be related to renal function data in order to ascertain whether more accurate prognostic indices can be developed and whether more sensitive measures of therapeutic response can be identified.

Project Description:

Objectives:

The classification of renal biopsy pathology in systemic lupus erythematosus (SLE) into general categories based mainly on the extent of cellular proliferation of glomerular cells as assessed by light microscopy has been a relatively poor predictor of prognosis in treated or untreated patients. Following immunologically mediated renal injury, renal function may improve by either regression of inflammatory changes in involved glomeruli or by compensatory increases of function in less involved glomeruli. The latter mechanism may explain why renal function is generally poor measure of cumulative renal damage until late in the chronic phases of nephritis when renal reserve capacity has been exhausted. Direct histological examination of renal biopsy material should provide a means to identify the extent of disease irrespective of the degree of renal functional compensation. The present study will examine the morphologic characteristics of SLE nephritis over time in patients being studied in ongoing therapeutic protocols. Biopsies will be scored for specific pathologic parameters which will be used as semi-quantitative indices of disease activity or chronicity. Repeat renal biopsy will be performed for evaluation of the same parameters at a specific time period, i.e. two years, regardless of the apparent clinical response of the patient to ascertain whether more accurate prognostic indicators can be identified and whether more sensitive measures of therapeutic response can be identified.

Methods Employed:

Percutaneous renal biopsy is performed under direct fluoroscopic visualization. Tissue samples are processed for routine light, immunofluorescence and electron microscopy. Sections are blinded and scored by both a nephropathologist and a clinical nephrologist on a checklist of specific morphological parameters.

Major Findings:

Clinical and routine laboratory studies may not identify patients with morphologically active disease as determined by renal biopsy.. Moreover, in those patients with clinically active renal disease, no specific parameter has been found to be predictive of the distribution nor the severity of pathologic lesions. We have found that patients with renal biopsies having a classification of diffuse proliferative glomerulonephritis have a broad spectrum of severity as judged by the type and number of active lesions either within glomeruli or in the interstitium. In addition the significance of sclerosing lesions, which may be concomitantly present with proliferation, appears to be an important prognostic factor which has not received adequate consideration in most series. Studies of serial renal biopsies using the semi-quantitative histological scoring system have been undertaken in patients who have entered a chronic stable phase of lupus nephritis. Preliminary results suggest that renal histologic changes are more severe in patients receiving corticosteroids alone compared to those receiving cytotoxic agents in addition. Analysis of larger numbers of patients is actively underway.

Significance to Bio-Medical Research and the Program of the Institute:

An improved system for evaluation and classification of renal biopsy pathology would provide the following advantages:

1. A clearer description of the types of pathology in SLE nephritis.

2. The ability to select more patients with more nearly homogenous types of nephritis for study of therapeutic responses.

3. Improved ability to identify accurate prognostic indicators of subsequent renal function during long-term therapeutic studies.

4. Provide an early index more sensitive than renal function as a measure of outcome in immunosuppressive drug trials in SLE nephritis.

5. Improved bases of indications for initiating and terminating therapy for SLE nephritis.

Proposed Course:

An ongoing study of patients who will be asked permission for repeat renal biopsy every two years is planned. Patients will be studied as they enter new therapeutic protocols as well as those followed in previous treatment programs.

Publications:

1. Balow, J.E.: Clinicopathological correlations in lupus nephritis, In Decker, J.L. (Moderator); Systemic lupus erythematosus: Evolving concepts. Ann. Intern. Med. 91: 587-604, 1979.

2. Balow, J.E., and Klippel, J.H.: Effects of immunosuppressive therapy on the histologic evolution of lupus nephritis. In, Schreiner, G.E. et al.(Eds.): Controversies in Nephrology. Vol. I. Washington, Georgetown Press, 1979, pp. 692-697.

3. Karsh, J., Klippel, J.H., Balow, J.E. and Decker, J.L. : Mortality in lupus nephritis. Arthritis Rheum. 22: 764-769, 1979.

4. Balow, J.E. and Klippel, J.H. : Immunosuppressive therapy in patients with advanced lupus nephritis. In. Schreiner, G.E. et al. (Eds.): Controversies in Nephrology. Vol. ii. Washington, Georgetown Press (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41039-03 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of cell mediated immune responses in normals and patients receiving immunosuppressive therapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J.E. Balow Senior Investigator ARB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of various immunosuppressive agents on lymphocyte functions is being studied to identify their sites and mechanisms of action. Various agents, such as cyclophosphamide and azathioprine are also being used as tools to study cell-mediated immune regulation in normal lymphocytes, as well as those from patients with autoimmune disorders, particularly SLE. The major goals of these studies are to further eludicate the mechanisms of altered immunoregulation, as well as to find new parameters of immune function to measure and/or monitor responses of patients receiving pharmacologic immunosuppressions.

PHS-6040

Project Description:

Objectives:

Abnormalities of immunoregulation are suspected to be the cause of several autoimmune disorders. Although some features of the complex cellular interactions involved in regulatory processes have been identified in normal subjects, studies of subjects with specific disorders and those receiving chemical immunosuppression have yielded important breakthroughs in our understanding of cells mediated immune regulation. The present studies will examine changes in immunologic function induced by immunosuppressive agents as well as by new experimental techniques.

Methods Employed:

1) Cell mediated cytotoxicity and lymphocyte proliferation assays will be predominantly used to evaluate the differential effect of cyclophosphamide on various subpopulations of lymphocytes from normal and patient donors. These studies will be performed by direct in vitro manipulation of lymphoid cells as well as by studies of changes induced by in vivo administration of these agents to patients with autoimmune disorders.

2) Studies of the role of circulating immune complexes in patients with autoimmune disease will be carried out. These will include studies of their role in the pathogenesis of glomerulonephritis, the effect of their removal by physical or chemical means, abnormalities of their clearance from the circulation in disease states (especially in glomerulonephritis), and studies of their possible effect on normal cell mediated immune responses.

Major Findings:

The in vivo administration of cyclophosphamide has been shown to augment T-lymphocyte function under certain conditions. Preliminary studies failed to show a differential sensitivity of immune regulator (suppressor) cells directly exposed to cyclophosphamide in vitro (Clin. Res. 27: 321A, 1979). These studies suggest that the enhancement of cell-mediated responses after in vivo cyclophosphamide results from a mechanism other than a direct inhibition of suppressor cells.

Significance to Bio-Medical Research and the Program of the Institute:

Characterization of the specific sites and mechanisms of action by various immunosuppressive agents would allow a greater selectivity in their clinical use for conditions associated with specific immunological defects. In addition, identification of their major site of action will hopefully lead to improvements in methods to monitor the desired effectiveness as well as the potential toxicity of these agents. Development of more selective means for correction of immunological abnormalities would obviously greatly enhance our care of clinical disorders.

Proposed Course:

This project has been "inactive" during the past year.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21040-08 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Study of various cytotoxic drug programs in diffuse lupus nephritis (PACIV)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D. Steinberg	Senior Investigator	ARB NIAMDD
	J.L. Decker	Chief, Arthritis and Rheumatism Branch	ARB NIAMDD
OTHER:	P.H. Plotz	Senior Investigator	ARB NIAMDD
	J.H. Klippel	Senior Investigator	ARB NIAMDD
	J.E. Balow	Senior Investigator	ARB NIAMDD
	H. Dinant	Guest Worker	ARB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with the diffuse forms of systemic lupus erythematosus nephritis are invited to enter a therapeutic trial. The three programs to which admissions continue are corticosteroids only, corticosteroids with cyclophosphamide and azathioprine both in low doses, and corticosteroids with intermittent intravenous cyclophosphamide.

Regular follow-up of the patients admitted continues and new patients are still being added.

Project Description:

Objectives:

The Arthritis and Rheumatism Branch has a major interest in the management of the nephritis of systemic lupus erythematosus (SLE). Renal failure is the most common cause of death in the disease. Deterioration of renal function, in some gradually and in others precipitately, does not seem to be much altered by adrenal corticosteroid therapy, a form of treatment which does control most of the symptomatic features. The renal damage is produced by immune complex deposition in the glomeruli. It has been suggested that drugs capable of altering the antibody response, "immunosuppressives," would reduce the formation of antibody and thus of antigen-antibody complexes. The objective of this study is to assess the efficacy and toxicity of various immunosuppressive drug programs in SLE nephritis.

Methods Employed:

Patients with systemic lupus erythematosus and active nephritis are invited to enter the trial. All patients are to be on prednisone and one group will receive no additional medications. The other two groups receive in addition: oral cyclophosphamide up to 1 mg/kg/day and azathioprine up to 1 mg/kg/day; and every 3 months intravenous infusions of cyclophosphamide up to 1 gm/M^2. All patients are repeatedly reevaluated with a battery of renal function tests, serological studies, urinalyses and, after two years in the trial, by renal biopsy when possible. It is proposed to continue the treatment programs indefinitely.

Major Findings:

Forty-one patients have been followed at least one year [13 on prednisone only (PO); 16 on cyclophosphamide plus azathioprine (CA), and 12 on IV cyclophosphamide (IC)]. Four patients have died -- none from renal failure or an abvious drug toxicity. Two patients stopped CA because of a desire to become pregnant and 2 stopped CA for drug side effects. Decline in renal function (serum creatine by 25%) was observed in 4 patients on PO, 2 patients on CA, and one patient on IC. Proteinuria and hematuria remained stable or improved in almost all patients.

Non-renal SLE flares occurred more commonly (x^2=6.1, $p<0.05$) in PO patients (13 in 39.2 observation-years) than in the other two groups (6 in 51.3 years with CA + 5 in 39.4 years with IC = 11 in 90.7 years).

Complications have included marked increase in asceptic necrosis in PO patients and increased herpes zoster in the other two groups.

Although decline in renal function has occurred somewhat more commonly in PO patients (4/12 = 33%) than in the two cytotoxic groups (3/27 = 11%), this difference has not reached statistical significance. Nevertheless, this encouraging trend coupled with fewer non-renal SLE flares in the IC and CA groups plus the increase in asceptic necrosis of bone in the PO group justifies continuation of the study.

Significance to Bio-Medical Research and the Program of the Institute:

These studies are relied upon by physicians concerned with SLE all over the world. We have been one of very few units able to speak from the hard data base of controlled studies. The net effect has been a great reduction in unwarranted enthusiasm for these agents. These studies certainly show that other modes of alleviating SLE renal disease are needed.

Proposed Course:

We will continue to follow these patients on their assigned programs. We are in the process of devising an accelerated program of intravenous cyclophosphamide to treat accelerated renal disease in patients with SLE.

Publications:

1. Klippel, J.H.: Studies in the treatment of lupus nephritis, In Decker, J.L. (Moderator): Systemic lupus erythematosus; evolving concepts. Ann. Intern. Med. 91:599-602, 1979.

2. Plotz, P.H., Klippel, J.H., Decker, J.L., Grauman, D., Wolff, B., Brown, B.C., and Rutt, G.: Bladder complications in patients receiving cyclophosphamide for systemic lupus erythematosus or rheumatoid arthritis. Ann Intern. Med. 91:221-223, 1979.

3. Steinberg, A.D.: Management of systemic lupus erythematosus. In Kelley, W.N., Harris, E.D., Ruddy, S., and Sledge, C.B. (Eds.): Textbook of Rheumatology. Philadelphia, W.B. Saunders Co., In Press.

4. Steinberg, A.D.: Immunoregulatory drugs. In Kelley, W.N., Harris, E.D., Ruddy, S., and Sledge, C.B. (Eds.): Textbook of Rheumatology. Philadelphia, W.B. Saunders Co., In Press.

5. Dinant, J.H., Klippel, J.H., Decker, J.L., Balow, J.E., Plotz, P.H., and Steinberg, A.D.: Randomized trial of oral cyclophosphamide plus azathioprine and intravenous cyclophosphamide in lupus nephritis. Arthritis Rheum. 23:666, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41041-02 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Aspirin and ibuprofen in SLE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	D. Magilavy	Clinical Associate	ARB NIAMDD
	D. Finbloom	Clinical Associate	ARB NIAMDD
	J.L. Decker	Chief, Arthritis and Rheumatism Br.	ARB NIAMDD
	J. Karsh	Guest Worker	CIB NIDR

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Treatment of the non-life-threatening manifestation of systemic lupus erythematosus is often aspirin or another non-steroidal anti-inflammatory drug. This study compares the efficacy and toxicity of aspirin and ibuprofen in some mild manifestation of SLE.

Project Description:

Objectives:

To compare the efficacy and toxicity of aspirin and ibuprofen for managing some manifestation of SLE.

Methods Employed:

After a brief control period, patients are given aspirin or ibuprofen in a double-blind fashion and their clinical response and laboratory tests are observed.

Major Findings:

Aspirin is slightly more effective than ibuprofen in controlling minor manifestation of SLE.

Significance to Bio-Medical Research and the Program of the Institute:

This study is one of several carried out by the Arthritis and Rheumatism Branch to improve the management of SLE.

Proposed Course:

Study completed

Publications:

1. Karsh J., Kimberly R.P, Stahl, N.I., Plotz, P.H., Decker, J.L. : Comparative effects of aspirin and ibuprofen in the management of systemic lupus erythematosus. Arthritis Rheum. (In press) 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41042-02 ARB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Intravenous Cyclophosphamide in the Treatment of Rheumatoid Arthritis (RA)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Klippel	Senior Investigator	ARB NIAMDD
OTHER:	J.L. Decker	Chief	ARB NIAMDD
	J. Karsh	Guest Worker	CIB NIDR
	P.H. Plotz	Senior Investigator	ARB NIAMDD
	N.L. Gerber	Chief	REHAB CC

COOPERATING UNITS (if any)

None

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The efficacy of intermittent intravenous cyclophosphamide in the treatment of rheumatoid arthritis will be evaluated in a double-blind randomized study. Patients unresponsive to conventional therapies will be assigned to receive a total of three intravenous infusions of either cyclophosphamide (1.0 gram per square meters, or less) or saline; one infusion every two months for a period of six months. The clinical activity of the arthritis and a functional assessment of patients will be quantitated during the study. Comparisons of the observed changes in these measures from assessments at the time of trial entry and two months after the final infusion will be made between treatment groups.

Project Description:

Objectives:

A number of controlled clinical trials have convincingly demonstrated the effectiveness of cyclophosphamide in the treatment of rheumatoid arthritis. However, the major factors limiting the clinical use of the drug in the treatment of RA is drug toxicity, including bone marrow depression, alopecia, amenorrhea or azospermia, bladder complications, infection and the potential for neoplasia.

Our experience with the use of intermittant, intravenous cyclophosphamide in the treatment of systemic lupus erythematosus suggests that this mode of drug administration may provide effective immunosuppression with minimal drug complications.

In this project, we propose to evaluate the efficacy and toxicity of intravenous cyclophosphamide given every two months in the treatment of patients with rheumatoid arthritis.

Methods Employed:

1. Patients with "active" rheumatoid arthritis unresponsive to conventional therapies will be randomized to receive three intravenous infusions of either cyclophosphamide or saline; one infusion every two months for a period of six months.

2. The clinical activity of the arthritis will be quantitated with an Articular Index measuring joint pain, tenderness and swelling.

Major Findings:

None at this time.

Significance to Bio-Medical Research and the Program of the Institute:

Cyclophosphamide is, perhaps, the most effective drug available for the treatment of rheumatoid arthritis. However, the drug toxicity associated with oral therapy is a major problem and limits drug administration.

In this project, we will attempt to determine the clinical efficacy in RA of presumed safer mode of administration of cyclophosphamide.

Proposed Course:

Patients satisfying the study adminssion criteria are being actively recruited.

Publications:

None at this time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41043-02 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunization of Patients with Systemic Lupus Erythematosus (SLE) with Pneumococcal Polysaccharide Vaccine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Klippel	Senior Investigator	ARB NIAMDD
OTHER:	J. Karsh	Guest Worker	CIB NIDR
	J.L. Decker	Chief	ARB NIAMDD

COOPERATING UNITS (if any)
Department of Microbiology and Immunology, Downstate Medical Center, Brooklyn, New York (G. Schiffman, Ph.D.)

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The immunogenicity and potential for disease modification of pneumococcal polysaccharide vaccine (Pneumovax) in SLE will be evaluated in a controlled, double-blind study. Patients will receive a single intramuscular dose of either vaccine or saline placebo. Anti-pneumococcal antibody concentrations to 12 type-specific pneumococcal capsular antigens will be measured by a radioimmune assay prior to and one and six months following vaccination. The patients will be monitored for clinical and laboratory abnormalities of SLE by a composite clinical, laboratory and serologic index.

PHS-6040

Project Description:

Objectives:

Infections are a major cause of morbidity and mortality in patients with systemic lupus erythematosus. The protection afforded by immunizations might be of benefit in this population provided that vaccines are immunogenic without resulting in exacerbations of the underlying disease.

In this project, we will evaluate the immunogenicity and clinical and serologic effects of a multivalent pneumococcal polysaccharide vaccine in SLE in a controlled, randomized study.

Methods Employed:

1. Patients will be randomized to receive 0.5 ml of intramuscular polyvalent pneumococcal polysaccharide vaccine or isotonic saline solution containing 0.25% phenol.

2. Serum will be collected prior to and one and six months after vaccination. Antibodies to each of 12 capsular polysaccharide types, measured by a radioimmunoassay, will be compared in vaccine and placebo-treated patients.

3. Clinical and laboratory measures of SLE activity will be monitored prior to and one and six months post vaccination to determine the effect of immunization on the underlying disease.

Major Findings:

1. One and six months following vaccination, vaccine-treated patients demonstrated significant increases in antibody concentration to each of 12 type specific capsular antigens as compared to placebo-treated patients. The serologic responses were specific for antigen comtained in the vaccine and unaffected by the administration of cytotoxic drugs cyclophosphamide or azathioprine.

2. Both pre- and post-vaccine antipneumococcal antibody concentrations in SLE patients were less than levels in normal healthy controls.

3. No clinical, laboratory or serologic effects on the underlying SLE were observed at one month following vaccination.

Significance to Bio-Medical Research and Program of the Institute:

In spite of effective antibiotic treatment, infections continue to be a major cause of mortality in patients with SLE. Thus, preventative measures, in particular the benefits achieved through immunizations, are of particular importance in this patient population. This project will evaluate the immunogenicity of a multivalent, bacterial vaccine in SLE.

Proposed Course:

All study patients will continue to be followed to determine if post-vaccination increases in antibody concentrations are maintained without unexpected late complications and if immunization results in a reduction in pneumococcal infections. An attempt will be made to determine whether observed differences in antibody responsiveness to specific antigens is on the basis of SLE disease activity, medications or HLA genetics.

Publications:

1. Klippel JH, Karsh J, Stahl NI, Decker JL, Steinberg AD, Schiffman G: A controlled study of pneumococcal polysaccharide vaccine in systemic lupus erythematosus. Arthritis Rheum 22:1321-1325, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41044-02 ARB

PERIOD COVERED

October 1, 1979 to September 31, 1980

TITLE OF PROJECT (80 characters or less)

Lymphocyte Depletion vs Plasma Removal in the Treatment of Rheumatoid Arthritis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.L. Decker	Chief	ARB NIAMDD
OTHER:	J.H. Klippel	Senior Investigator	ARB NIAMDD
	J. Karsh	Guest Worker	CIB NIDR
	D.G. Wright	Senior Investigator	POB NCI
	A.B. Deisseroth	Chief	POB NCI
	W. Flye	Senior Investigator	SURG NCI

COOPERATING UNITS (if any)

Clinical Immunology Branch, NIDR; Pediatric Oncology Branch, NCI; Surgery, NCI

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Diseases

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with rheumatoid arthritis unresponsive to conventional therapies will be offered participation in a double-blind therapeutic trial. They will be randomized to two treatment protocols comparing the effects of lymphocyte depletion (lymphapheresis) and plasma removal (plasmapheresis) on rheumatoid synovitis. The disease activity will be monitored by an Articular Index. The risks and benefits of each treatment will be evaluated and compared at the end of 5 weeks of therapy and through a 6 to 12 month follow-up period.

PHS-6040

Project Description:

Objectives:

A previous pilot study (Clinical Project 76-A-25) suggested that lymphocyte depletion may benefit patients with severe rheumatoid arthritis. The process of lymphocyte depletion (lymphapheresis) involved not only the removal of lymphocytes but plasma, polymorphonuclear cells and platelets as well. Although circulating levels of serum albumin, polymorphonuclear cells and platelets did not change it is possible that temporary removal of serum factors and/or the above cells may have accounted for the beneficial effects. The goals of the study are to compare the relative benefits of cell depletion and plasma removal under identical circumstances.

Methods Employed:

Twelve patients meeting predefined criteria for severe recalcitrant rheumatoid arthritis will be admitted to the trial and randomly assigned to either lymphapheresis or plasmapheresis. To ensure repetitive venous access arterio-venous fistulae will be constructed at the wrist. Upon fistula maturation patients will be re-admitted to undergo 15 procedures over a 5 to 6 week study period. Entry medications will be limited to non-steroidal anti-inflammatory drugs and prednisone and will be held constant for the preceding month, the period of treatment, and the follow-up period until relapse has occurred. A single examinor unaware of the nature of the patient's therapy will perform serial assessments of rheumatoid activity. Routine hematological, chemical and serological parameters will be followed. In addition special studies will follow the peripheral lymphocyte count and the distribution of lymphocyte sub-types in the circulation.

Major Findings:

The study has been completed and reported to the American Rheumatism Association. Six patients receiving lymphapheresis demonstrated statistically significant decrements in counts of active joints and swollen joints and in a graded articular index, as compared to 6 patients similarly treated by the control procedure restricted to the removal of the plasma volume incidentally lost during lymphapheresis. No significant untoward effects were observed. The magnitude of the change in synovitis did not correlate with the number of mononuclear cells removed nor with the percent reduction in circulating lymphocytes, T lymphocytes, or serum IgM. The mechanism of the effect remains obscure.

Significance to Bio-Medical Research and Program of the Institute:

The work has provided critical insights on the difficulties of quantitating disease activity in rheumatoid arthritis. It also points the way for further studies of the modality which may very well become a practical method of treating severely ill patients whose disease has not responded to more conventional treatment plans.

Proposed Course:

A follow-up study directed toward the potential role of monocyte removal is in the planning stages.

Publications:

None at this time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41048-01 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Study of Genetic and Pathophysiologic Features in the Rat of Experimental Erosive Synovitis Induced by Bacterial Cell Walls

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.L. Wilder	Clinical Associate	ARB NIAMDD
Other:	G.B. Calandra	Senior Investigator	LSD NIAID
	J.G. Garvin	Senior Investigator	LP NCI
	C.T. Hansen	Chief	VR RR
	L.M. Wahl	Senior Investigator	LMI NIDR
	J.L. Decker	Chief	ARB NIAMDD
	J.H. Klippel	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)
Laboratory of Streptococcal Diseases, NIAID; Laboratory of Pathology, NCI; Veterinary Resources Branch, RR; Laboratory of Microbial Immunology, NIDR

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Erosive synovitis can be induced experimentally in rats by intraperitoneal injection of bacterial cell walls from group A streptococci. Genetic factors determine susceptibility to development of disease. High responder inbred rat strains develop marked mononuclear cell inflammation, most evident peripheral joints. Low responder inbred strains show little evidence of pathology. This model may facilitate characterization of factors that potentiate the development of chronic destructive arthritis.

PHS-6040

Project Description:

Objectives:

Published data show that experimental erosive synovitis can be elicited in rats by intraperitoneal injection of cell walls from certain groups of streptococci. Studies in progress have the following goals:

1. To define the variability in incidence, type and severity of the experimental arthritis.

2. To define the properties of bacteria that facilitate development of arthritis.

3. To characterize the host genetic factors which determine susceptibility to arthritis.

4. To better define pathophysiologic processes by characterizing differences between high and low responder inbred rat strains.

Methods Employed:

1. Bacterial cell walls, free of membrane, are isolated from Streptoccoccus pyogenes, Group A, solubilized by sonication, and used to induce arthritis in various outbred and inbred rat strains.

2. Rats, both inbred and outbred, are injected intraperitoneally with the cell wall preparations.

3. The rats are observed frequently to document the time of onset severity and characteristics of the arthritis and other clinically evident pathology. Additional assessment is made by radiography and histology.

4. Selected inbred strains are used in breeding studies to determine the importance of selected genetic determinants such as the major histocompatibility (MHC) and Ig allotype complexes.

5. Selected strains are analyzed in terms of macrophage and lymphocyte function

Major Findings:

1. More than 50 outbred sprague-dawley rats have been studied. About 30-40% develop a distal destructive arthritis with variable severity. These results suggest genetic variability influences the development of disease.

2. Fourteen inbred rat strains have been studied. A genetic predisposition to disease has been unequivocally demonstrated. Several strains are 100% susceptible; several others appear markedly resistant.

3. The characteristic pathologic lesion is a chronic mononuclear cell infiltration consisting of macrophages and lymphocytes in addition to marked synovial cell proliferation.

4. Disease susceptibility appears to be determined by several genetic loci, including non-MHC genes.

5. Development of arthritis appears to depend on the persistence of cell wall materials for prolonged periods of time in the affected tissue.

Significance to Bio-Medical Research and Program of the Institute:

Rheumatoid disease and several other conditions characterized by chronic synovitis are suspected to result from prolonged inflammation induced by an unknown substance in an individual genetically predisposed to this type of inflammation. The experimental model we are studying demonstrates that this theory is reasonable and suggests possible important pathophysiologic mechanisms. This model may allow us to derive more rational means of diagnosing and treating arthritis in man.

Proposed Course:

The genetic and pathophysiologic characterization of this experimental model is continuing.

Publications:

None at this time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41049-01 ARB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The comparative efficacy and safety of azathioprine and D-penicillamine for the treatment of rheumatoid arthritis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	J. L. Decker	Chief, Arthritis & Rheum. Br.	ARB NIAMDD
	J.H. Klippel	Senior Investigator	ARB NIAMDD
	R. Wilder	Clinical Associate	ARB NIAMDD

COOPERATING UNITS (if any)

none

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

There are few therapeutic options open for the treatment of patients with severe rheumatoid arthritis who have have failed to respond to gold or who have developed an unacceptable toxicity. In this trial, the comparative efficacy and safety of two drugs widely used in this situation are being compared. D-penicillamine (recently approved by the FDA for treatment of RA) and azathioprine, a cytotoxic drug used widely for the treatment of various connective tissue diseases but not yet approved for rheumatoid arthritis. We are one of 11 cooperating clinics in this trial.

PHS-6040
(Rev. 10-76)

Objectives:

To determine the comparative efficacy and safety of azathioprine and D-penicillamine in the treatment of rheumatoid arthritis in patients who have failed to respond to gold or who have developed unacceptable toxocity to it

Methods Employed:

Double-blind trial

Major Findings:

None yet

Significance:

The careful study of the comparative efficacy and safety of these drugs will allow more intelligent use of them in the future.

Proposed Course:

Admission of up to 10 more patients in the coming year.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41050-01 ARB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Comparative efficacy and safety of myochrysine and auranofin in the treatment of rheumatoid arthritis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	J.L. Decker	Chief, Arthritis & Rheum. Br.	ARB NIAMDD
	J.H. Klippel	Senior Investigator	ARB NIAMDD
	R. Wilder	Clinical Associate	ARB NIAMDD

COOPERATING UNITS (if any)

none

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Gold has been known to have a beneficial effect on rheumatoid arthritis for many decades. In this study, a new, gold preparation that can be taken by mouth will be compared to a placebo and to intramuscular gold in a double-blind trial involving 11 clinics cooperating under the aegis of the Center for Cooperating Studies in the Rheumatic Diseases.

PHS-6040
(Rev. 10-76)

Objectives:

To determine the comparative efficacy and safety of two forms of gold and placebo in the treatment of rheumatoid arthritis

Methods Employed:

A double-blind drug trial with 3 groups of patients.

Major Findings:

None yet

Significance:

To find a form of gold of comparable efficacy and sagety to standard intramuscular gold which can be taken by mouth would be a useful advance in the management of rheumatoid arthritis.

Proposed Course:

To continue to enter up to 10 patients in the coming year.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41051-01 ARB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Title: Natural History and in situ organization of the receptors for IgE.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI C. Isersky-Carter, Senior investigator ARB, NIAMDD

Others H. Metzger, Chief, Section on Chemical Immunology ARB, NIAMDD
T. Triche, Chief, Section on Ultra Path. LP, NCI

COOPERATING UNITS (if any)

D. Segal I.B. NCI, E. Barsumian and R. Siraganian LMI, NIDR.

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Chemical Immunology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are investigating the natural history and in situ organization of the receptor for IgE on mast cells and basophils. Various sublines of rat basophilic leukemia (RBL) cells and plasma membrane vesicles prepared from them are used. Purified IgE myeloma, antibodies to IgE and to the receptor are also used. These studies will help clarify the mode of function of the receptor, the fate of the receptor-bound monomeric and cross-linked IgE and the turnover of the free receptor.

Project Description

Objectives

The various rat basophilic leukemia cell lines as well as purified labeled IgE myeloma and hybridoma provide a unique model system with which one can follow the fate of a protein that binds specifically and tightly to a surface receptor. The main objectives are to investigate the conditions under which the surface bound IgE is internalized and the effect of internalization on the turnover of the receptor. These cell lines also provide a source of plasma membrane vesicles carrying purified radiolabeled marker for the outer leaflet of the plasma membrane, namely IgE. The main objective here is to elucidate the manner in which the receptor is organized in the membrane, and thus learn about the mechanism involved in transmitance of signals across the cell membrane.

Major findings

A. The fate of IgE bound to rat basophilic leukemia cells.

I. Monomeric IgE.

Both histamine-releasing and non releasing cell lines were examined. In both cases, no evidence for cell-mediated IgE catabolism could be elicited. Both the dissociated IgE and the receptors remained intact for prolonged periods of time, as demonstrated by binding assays. Internalization and/or recycling of membrane-bound IgE could not be demonstrated by E.M. autoradiography. We found only limited time-dependent changes in accessibility to anti-IgE antibody, trypsin, or elution at low pH (2.9 to 3.1). A biphasic dissociation of cell-bound ^{125}I-IgE during incubation in the presence of excess unlabeled IgE was reproducibly observed; the more slowly dissociated IgE was also less readily dissociated at pH 3.4. Cell bound IgE was most significantly less sensitive to trypsin than free IgE. These studies lead us to conclude that, in vitro, IgE resides in a functional orientation on the surface of RBL-1 cells, for prolonged periods of time. Among immunoglobulins IgE has the shortest serum survival and the highest catobolic rate. Yet passively sensitized mammals remain reactive to allergens for many years. In view of our findings this is no longer as much of a paradox since membrane binding can impart to the IgE diminished sensitivity to catobolism, thus the cell bound IgE remains available to allergens for prolonged periods of time.

II. Oligomeric IgE.

Both histamine-releasing and non-releasing cell lines were examined. IgE dimers, trimers and heteromers were all

internalized by the histamine releasing cells whether or not they were shown to be effective in bringing about exocytosis. The non-releasing cell lines internalized dimers and trimers as well as the releasing cell lines. While 35 to 70% of the surface-bound oligomers were internalized this was not associated with exposure of new receptor sites. The histamine releasing cells express IgG receptors capable of binding IgG and IgE oligomers, bound IgG oligomer was internalized. Since the binding of either IgE or IgG oligomers is inhibited in presence of excess homologous monomer we conclude that binding is specific and internalization is receptor driven. Importantly the binding of a dimer of IgE imparted a signal leading to internalization.

B. Preparation and characterization of plasma membrane vesicles.

We have isolated membrane vesicles from RBL cells. This was achieved by gradual hyptonic swelling of cells followed by Dounce homogenization, separation on (20-50%) sucrose gradient, sonication and centrifugation (50,000xg). The resulting membrane preparation was morphologically (E.M.) homogenous and when banded in sucrose gradient peaked at 26-31%. The overall yield was > 50%, the specific activity of membrane bound ^{125}I-IgE increased 20 fold, and 99.8% of the DNA and 97% of the RNA present in the homogenate, were eliminated. The vesicle preparation did not bind the expected amounts of additional IgE. When passed through anti-IgE affinity columns 25-80% of the vesicles in various preparations were not retarded. Some of the preparations were assessed on con A affinity columns and similar results were obtained. Solubilization in non-ionic detergent resulted in recovery of the expected capacity of the free receptor to bind IgE and of the receptor associated IgE to bind to anti IgE affinity columns. Whether or not these represent 'reversed' vesicles would require more studies using an E.M. identifiable outer membrane marker such as an IgE-Ferritin complex.

C. Characterization of various rat basophilic leukemia cell lines. (I -IV)

RBL-I and III have ~44 chromosomes and did not respond to an IgE-mediated reaction, whereas RBL-II and RBL-IV have 68-73 chromosomes and showed moderate levels of histamine release (percent release $\bar{x}$ = 5 $\pm$ 2 and 10 $\pm$ 4 respectively). The cloning of the RBL-IV line resulted in some sub-lines which were excellent histamine releasers (range 39% to 100%) and some which were relatively refractory (<10%) to IgE-mediated histamine release. These clones did not differ significantly in chromosome number. Recloning the releasing lines gave rise to poor releasers, whereas, the recloning of poor-releasers did not produce good releasers, indicating that the mutational drift in culture is toward loss of histamine releasing capacity. The number of IgE receptors, as well as the rate of IgE association and dissociation were similar for the different cell

lines. The study failed to disclose significant molecular weight differences in the IgE-receptor from the various clones and sublines, but disclosed a multicomponent peak in SDS-PAGE analysis of receptors extracted from all the cloned sublines. This indicates that the multicomponent broad peak can not be due to heterogeneity of cells. The results indicate that the cell lines probably do not differ at the receptor level. The various cloned sublines are phenotypically stable and therefore useful for studies of the complex phenomenon of histamine release.

Publications

1. C. Isersky, J. Rivera, S. Mims and T.J. Triche: The fate of IgE bound to rat basophilic leukemia cells. J. Immunol. 122: 1926-1936, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41052-01 ARB

PERIOD COVERED October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Disorders of immune regulation in patients with systemic lupus erythematosus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. E. Balow	Senior Investigator	ARB NIAMDD
Other:	G. C. Tsokos	Visiting Fellow	ARB NIAMDD

COOPERATING UNITS (if any)

none

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with systemic lupus erythematosus have been found to have disturbances of cell-mediated immune responses. Alterations of B and T lymphocytes as well as monocyte function have been observed both in patients with SLE and certain of their relatives. The major goal of these studies is to further characterize the types of immunological disturbances in SLE and to elucidate the mechanisms which lead to alterations of immune regulation and are responsible for the pathogenesis of this disease.

Project Description:

Objectives

SLE is considered to be caused by disturbances in immune regulation. Although several disorders of the T and B lymphocyte functions have been noted in patients with SLE, certain of these abnormalities: 1) are absent in some affected SLE patients, 2) continue to be present after patients enter remission of SLE, and 3) have also been observed in non-affected relatives of SLE patients. Thus, the questions regarding which abnormalities are necessary and/or sufficient for the pathogenesis of SLE remain unclear. The present studies seek to elucidate certain of the mechanisms causing abnormal cell-mediated immunological dysfunction in order to further understand the pathogenesis and to consider novel approaches to therapy of SLE.

Methods Employed:

1. B lymphocyte functions will be studied by enumeration of immunoglobulin secreting cells in reverse hemolytic plaque assays. Various subpopulations of lymphoid cells from SLE patients will be tested in combination in order to elucidate the types of cells with either excessive or deficient immunoregulatory function.

2. T lymphocyte functions will be studied in assays of cell-mediated cytotoxicity, mixed leucocyte reaction and lymphocyte proliferation in response to various in vitro mitogenic stimuli. The role of autoantibodies directed against selected lymphocyte subpopulations, as well as the role of circulating immune complexes, in causing disturbances of immune regulation will be studied.

3. Studies of cellular interactions involving T and B lymphocytes and monocytes will be undertaken to further determine the state of helper and/or suppressor cell subsets in patients with SLE as compared to those of normal subjects.

Major Findings:

Patients with SLE have been found to have elevated numbers of spontaneously activated immunoglobulin secreting cells in their peripheral leucocytes. Patients with the most clinically active disease have the highest proportion of activated B lymphocytes which, however, do not respond normally to stimulation with polyclonal activators, such as pokeweed mitogen. However, a radiation-resistant accessory lymphoid cell from normal donors partially restores this defective response to B cell mitogens (Clin. Res. 28: 364A, 1980). These studies suggest that patients with SLE may lack a helper cell function necessary for normal B lymphocyte response, or that prior activation of B lymphocytes in vivo leads to refractoriness to mitogenic stimuli. Alternatively, the emergence of suppressor cell activity following in vivo B lymphocyte activation may lead to inhibition of immunoglobulin secretion.

Deficiency of T cell functions have been found in patients with SLE. Certain patients lack the ability to generate cytotoxic T lymphocytes to alloantigens, while nearly all patients exhibit a normal proliferative response to alloantigens in mixed leucocyte culture (Clin. Res. 28: 362A, 1980). Preliminary experiments suggest that the defect in ability to generate cytotoxic T lymphocytes may be caused by pre-activated suppressor cells in certain patients with SLE.

Significance to Bio-Medical Research and the Program of the Institute

The complex interacting and regulatory network of the normal immune response may reflect perturbations several cell levels distant from the primary disturbance. Clarification of the mechanisms causing each immunologic disturbance in SLE is necessary in order to ascertain their relative importance in the pathogenesis of SLE.

Proposed Course:

Continued studies of cell mediated lymphocyte functions of patients with SLE in various levels of disease activity in comparison to those of normal controls will be undertaken.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41053-01 ARB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Effects of High-Dose Intravenous Methylprednisolone on the Course of Rheumatoid Arthritis (RA)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.L. Wilder	Clinical Associate	ARB NIAMDD
Other:	J. Karsh	Guest Worker	LMI NIDR
	J.H. Klippel	Senior Investigator	ARB NIAMDD
	J.L. Decker	Chief	ARB NIAMDD
	A.S. Fauci	Senior Investigator	LCI NIAID
	P.H. Plotz	Senior Investigator	ARB NIAMDD
	N.L. Gerber	Chief	REHAB CC

COOPERATING UNITS (if any)

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Six patients with rheumatoid arthritis unresponsive to conventional therapies have been studied in an open pilot trial of short-term pulse methylprednisolone (MP). Following administration of 1 gram of MP on 3 consecutive days, dramatic changes in articular index were evident in the first 1-2 weeks. The effect persisted for a variable period of time - 2 weeks to more than 8 weeks. No major side effects were observed. No persistent changes in several immunological parameters were measured. The results suggest further study of this form of therapy is warranted.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Although corticosteroids are dramatically effective in diminishing inflammation, their administration to patients with rheumatoid arthritis on a prolonged daily basis is often associated with severe complications. Administration of steroids by intermittent high dose "pulses" is frequently effective in renal transplant rejection, whereas administration by conventional regimens are usually ineffective. This mode of administration has been generally safe. Because we suspect that transplant rejection and rheumatoid synovitis may have some cell-mediated inflammatory mechanisms in common, an open pilot study of high dose pulse methylprednisolone (MP) therapy was initiated. Objectives of the study were:

1. To determine if short-term pulsed intravenous (IV) MP could induce significant improvement of subjective and objective signs of inflammatory arthritis in patients with rheumatoid arthritis.

2. To determine the nature of an duration of any beneficial effects.

3. To document incidence, type, and severity of reactions of this mode of treatment.

Methods Employed:

1. Patients admitted to the study met criteria for definite or classic RA, had persistently active disease, and had failed to adequately respond to non-steroidal anti-inflammatory agents and at least 2 other major anti-rheumatic drugs (gold, antimalarials, penicillamine or cytotoxic) drugs. Cytotoxic drugs, if previously tried, were discontinued 2 months prior to entering the study. Other drugs, including low dose corticosteroids, were maintained on a stable regimen for 6 months prior to pulse treatment.

2. The study consisted of 3 phases: a) pre-pulse MP evaluation (one month) to establish baseline parameters; b) pulse MP administration (5 days in hospital); single three-day course of IV MP, 1 gm/day; c) post-pulse MP evaluation (two months).

3. Each patient was assessed by a single examiner while in the study.

4. A variety of chemical, hematological and immunological parameters were assessed.

Major Findings:

1. All patients showed dramatic improvement measured by multiple parameters at one week. The improvement lasted for a variable period of time - 2 weeks (one patient) to more than 8 weeks (three patients).

2. No major side effects were observed. Minor side effects included transient insomnia, increased dreaming, flushing and a metallic taste.

3. No persistent changes were noted in chemical, hematological or immunological parameters studied, except for a decrease in erythrocyte sedimentation rate.

Significance to Bio-Medical Research and Program of the Institute:

Therapy for severe rheumatoid arthritis remains controversial and often inadequate. The dramatic results in this pilot study suggest that further study of this form of steroid administration as an adjunct to other therapies is warranted.

Proposed Course:

Further studies are being discussed.

Publications:

None at this time.

METABOLIC DISEASES BRANCH

The general goals of the Branch are to investigate the structure, secretion and biochemical mechanism of action of hormones governing ion transport and mineral metabolism and to apply the knowledge gained to understanding and treating related clinical disorders. Current investigations include hormone-receptor interaction, mechanism of receptor and guanine nucleotide control of adenylate cyclase, cyclic AMP control of ion transport, and regulation of parathyroid hormone secretion. Systems under study include β-adrenergic receptors in several tissue, isolated parathyroid cell systems and skeletal and renal tissue. Clinical studies on hyperparathyroidism and pseudohypoparathyroidism continue to utilize extensively the research findings from the laboratory. Radioimmunoassays for parathyroid hormone have been applied to determine the hormone in samples obtained selectively by venous catheterization, thereby allowing localization of parathyroid adenomas. Further studies make use of urinary cyclic AMP clearance as a clinical parameter of parathyroid hormone action. Another investigation takes advantage of the autologous parathyroid transplant program developed in collaboration with the Surgery Branch, NCI. Autologous transplants to the arm allow direct sampling of draining veins for parathyroid hormone secretion in man under conditions wherein the tissue can be selectively challenged with potential secretagogues or inhibitors of parathyroid secretion.

β-Adrenergic Receptor of the Avian Erythrocytes.

Interactions of catecholamines with β-adrenergic receptors and activation of adenylate cyclase are under study with the plasma membranes of turkey erythrocytes and other cell systems. Specific receptors have now been identified on turkey erythrocytes, parathyroid cells, pineal cells, rat, guinea pig and monkey lung membrane preparations and rat liver membranes.

β-Adrenergic receptor sites are identified using a radioiodinated β-blocker iodo-hydroxybenzylpindolol. A series of β-adrenergic agonists and inhibitors compete for binding with affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. Receptor occupancy for agonists correlates linearly with function (cyclic AMP production). The stereospecificity of interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological effectiveness indicate that binding of iodohydroxybenzylpindolol validly reflects interaction with the β-adrenergic receptor.

Calcitonin receptors have been identified and studied in several tissues wherein they regulate cyclic AMP-mediated processes. Although calcitonin polypeptides have undergone remarkable evolutionary changes in structure and effectiveness, there has been no significant corresponding change in biological nature of calcitonin receptors.
[Drs. Marx and Aurbach, NIAMDD]

Guanine Nucleotide Regulatory Protein

Studies on the role of guanine nucleotides in the control of adenylate cyclase activity are continuing. Guanine nucleotides enhance catecholamine-stimulated adenylate cyclase activity in turkey erythrocyte membranes. This action is mediated by a guanine nucleotide binding regulatory protein which is distinct from the receptor and the catalytic unit of adenylate cyclase. In the turkey erythrocyte membrane system, exchange of guanine nucleotides bound to the regulatory protein is strictly dependent on the action of hormone (β-adrenergic agonists). However, treatment of membranes with isoproterenol and GMP (a nucleotide bound with low affinity) permits later hormone-dependent activation and manipulation of the regulatory protein. After isoproterenol and GMP treatment, the guanine nucleotide regulatory protein is extracted from the membrane by Lubrol PX, and adsorbs to a GTP affinity matrix, from which it is eluted by high concentrations of guanine nucleotides. The regulatory protein, purified 50-100 fold by affinity adsorption, restores both guanine nucleotide- and fluoride-responsive adenylate cyclase activity to membranes from a mutant (CYC^-) strain of S49 mouse lymphoma which lacks the regulatory protein.

The guanine nucleotide regulatory protein from turkey erythrocytes is covalently radiolabeled by the cholera toxin-catalyzed transfer of [^{32}P]ADP-ribose from [^{32}P]NAD to a 42,000 M_r subunit of the regulatory protein. [^{32}P]ADP-ribosylation permits detection of the regulatory protein during detergent extraction, affinity purification, sucrose gradient centrifugation, and manipulations which cause inactivation of the regulatory protein. Radiolabeling of the regulatory protein should permit more accurate quantitation of enrichment during purification. Similar techniques applied to human tissue may permit detection of quantitative and qualitative alteration in hormone-resistant states such as pseudohypoparathyroidism.

Changes in the nature of the guanine nucleotide bound to the regulatory unit influence fluoride-stimulated adenylate cyclase activity. Incubation of turkey erythrocyte membranes with isoproterenol and GMP clears many of the regulatory sites of tightly bound GDP, substituting GMP. Fluoride-stimulated adenylate cyclase activity declines during isoproterenol and GMP treatment, but is restored by addition of GTP or GDP. Studies performed with the GDP analogs GP(CH)$_2$P and GDP-β-S, and direct analysis of the nucleotides bound at isoproterenol-dependent regulatory sites during fluoride activation, indicate that fluoride does not enhance conversion of GDP to GTP at regulatory sites, nor does it inhibit hydrolysis of GTP to GDP. Also, exchange of guanine nucleotide bound to the regulatory protein with nucleotides in the medium does not occur during enzyme activtion by fluoride.

Studies with partially purified, affinity eluted, guanine nucleotide regulatory protein confirm that fluoride stimulation of adenylate cyclase depends on the guanine nucleotide bound to the regulatory unit. Regulatory protein eluted with either GTP or GDP restores fluoride-stimulated enzyme activity to the S49 CYC^- acceptor, but regulatory protein eluted with GMP restores fluoride-stimulated activity only when GTP or GDP were added, indicating that the guanine nucleotide regulatory protein, and an appropriate nucleotide, are necessary for fluoride-stimulated adenylate cyclase activity. [Drs. Spiegel, Downs, Attie, Levine and Aurbach, NIAMDD]

The molecular defect in pseudohypoparathyroidism (PHP) has been investigated using techniques adapted from basic studies on the turkey erythyrocyte adenylate cyclase complex. The guanine nucleotide regulatory unit (G unit) in red cell membranes from patients with PHP was assayed and found to be reduced by ∿50% compared to controls. This finding suggests that a reduction in functional G units involving all cell types may account for hormone resistance in this disorder.
[Drs. Spiegel, Levine, Downs, Marx and Aurbach]

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case finding has produced 41 kindreds for analysis. These studies have allowed segregation of the familial variants into two apparently distinct disease syndromes - familial multiple endocrine adenomatosis (MEN I) and familial hypocalciuric hypercalcemia (FHH). FHH was distinguished from MEN I by 1) virtually a 100% penetrance for hypercalcemia before age 20, 2) milder clinical manifestations - low incidence of recurrent nephrolithiasis or recurrent peptic ulceration, 3) no hypercalciuria, 4) normal basal concentrations of gastrin, and 5) poor response to subtotal parathyroidectomy. Distinction between the two syndromes, both inherited as autosomal dominant traits, is important because in FHH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial. FHH accounts for approximately 10% of all unsuccessful parathyroidectomies in hypercalcemia. In FHH the ionized and ultrafiltrable calcium concentration in serum is elevated in proportion to the increase in total calcium. In these patients the filtrable load of calcium is high in association with a marked decrease in renal calcium clearance. The concentration of parathyroid hormone in plasma is lower in patients with FHH than in typical primary hyperparathyroid patients with similar degrees of hypercalcemia whether assessed by PTH radioimmunoassay or by renal clearance of cAMP or phosphate. The plasma and urine cAMP response to PTH infusion is greater in FHH than in typical primary hyperparathyroidism. Even when these patients become surgically hypoparathyroid, the low renal clearance of calcium is strikingly persistent during calcium infusion.
[Drs. Marx, Spiegel, Downs, Attie, Levine and Aurbach]

Studies on modes of localizing parathyroid tumors continue. A high degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded, in approximately 45% of cases tested, the identification of abnormal masses of tissue subsequently identified as parathyroid. Confirmation of the mass thus identified as parathyroid tissue can be accomplished by verifying high concentrations of parathyroid hormone by radioimmunoassay in veins draining the lesion. Draining vessels contain concentrations of hormone 2- to 20-fold or more greater than the concentration of parathyroid hormone detected by immunoassay in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in the one vein draining the lesion. This technique utilizing specific radioimmunoassays and selective catheterization is applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by non-parathyroid cancers. Localization of anterior mediastinal parathyroid adenomas has become possible using computed tomography. Eight mediastinal adenomas have been treated nonsurgically by percutaneous injection via catheter

occlusive agents into the arterial blood supply with 7 complete and one partial remissions.

Although results with parathyroid radionuclide imaging after intravenously administered [^{75}Se]-selenomethionine have been disappointing, we have been obtaining parathyroid scans after administering this isotope intra-arterially during parathyroid arteriography. Several cases wherein cannulation of most of the important vessels potentially supplying the parathyroids was possible have shown correctly localizing scans although careful interpretations are necessary to avoid false positives. This technique appears to be especially useful in those patients with juxta- or intra-thyroidal adenomas even when arteriography is ineffective. Radionuclide images are often positive because suppression of the thyroid with triiodothyronine prior to scanning improves the specificity of the parathyroid scan but not of the arteriogram.
[Drs. Marx, Spiegel, Downs, Stock and Aurbach, NIAMDD; Drs. Doppman, Jones and Krudy, Radiology Department, C.C.]

We have studied a patient with parathyroid carcinoma who showed markedly elevated serum concentrations of the glycoprotein hormone subunits hCG-α and hCG-β, which fell dramatically after surgical removal of malignant tissue. Screening of a large number of patients with different forms of hyperparathyroidism has shown these subunits to be a marker for malignancy in parathyroid disease or for islet cell involvement in familial multiple endocrine adenomatosis (MEN I).
[Drs. Marx, Spiegel, Downs, Stock and Aurbach, MDB, NIAMDD; Drs. Weintraub and Rosen, CEB, NIAMDD]

The role of $1,25(OH)_2D_3$, the most potent natural metabolite of cholecalciferol, has been assessed in hypocalcemic states. We have discovered a family with a unique disturbance of calciferol metabolism. Two siblings have rickets and osteomalacia attributable to decreased target tissue sensitivity to $1,25(OH)_2D$. When maintenance of serum concentrations of $1,25(OH)_2D$ with appropriate calciferol analogs at concentrations approximately 20-fold normal, allows full control of the disorder. One of these patients was monitored during the course of her first pregnancy. The fetus developed normally even though $1,25(OH)_2D_3$ levels were extraordinarily high in maternal and cord blood.
[Drs. Marx, Spiegel, Downs, Eil, Hamstra and DeLuca]

Rapid determination of intraoperative U_{cAMP} excretion (using the Gammaflo machine for <7 min cAMP radioimmunoassay) has proven to be a valuable tool in guiding surgery for primary hyperparathyroidism. Persistent elevation of U_{cAMP} requires continued search for abnormal tissue even after 1 or more abnormal glands have been removed. A rapid (mean 1.5 hours) drop in U_{cAMP} to the normal range obviates the need for continued exploration even in cases where histologic confirmation of parathyroidectomy is lacking. Spurts in U_{cAMP} above baseline may provide a clue to the location of abnormal parathyroid tissue.
[Drs. Spiegel, Marx, Downs, Attie, Stock, Aurbach, NIAMDD; Saxe and Brennan, NCI Surgery]

Determination of urinary cAMP excretion postoperatively in patients undergoing neck exploration for primary hyperparathyroidism is a useful method for assessing postoperative parathyroid function. UcAMP excretion declines postoperatively in all patients in whom hypercalcemia is corrected but not in those with persistent hypercalcemia. In patients becoming severely hypocalcemic (and requiring vitamin D therapy) postoperatively, UcAMP measurement enables one to distinguish patients with decreased parathyroid reserve as the cause for hypocalcemia (low UcAMP excretion) from patients with healing osteitis fibrosa ("hungry bones") as the basis for hypercalcemia. UcAMP in the latter group is often elevated but can be suppressed if serum calcium is normalized. Elevated UcAMP excretion postoperatively in the face of hypoe calcemia enables one to predict that vitamin D therapy will be required temporarily and precludes the need for parathyroid autografts.
[Drs. Spiegel, Marx, Downs, Attie, Levine and Aurbach, NIAMDD]

Studies have continued with dispersed bovine and human parathyroid cells. PTH secretion from parathyroid glands in vivo and cells in vitro is inhibited by calcium. Calcium may also modulate synthesis and degradation of PTH. This control exerted by calcium may be changed in certain pathologic states (glandular adenomas, carcinomas and perhaps hyperplasia). Several earlier studies have strongly suggested that cAMP is an intracellular mediator of PTH secretion; agents which alter cellular cAMP change PTH secretion in the same direction. Calcium decreases cellular cAMP although some of its effects may be independent of cAMP. The adenylate cyclase of the parathyroid cell is a potential regulatory site for the action of calcium. Adenylate cyclase in homogenates of dispersed parathyroid cells shows sensitivities to Ca (1/2 maximal inhibition at approximately 0.2 mM added calcium) that are similar in human parathyroid adenomas, carcinomas or hyperplastic glands and like that of normal bovine glands. There was no correlation between the in vitro calcium suppressibility of PTH release by dispersed cells (several of the adenomas and the one carcinoma were not suppressed by high extracellular calcium) and the sensitivity of the adenylate cyclase in the broken cell preparation. This suggests that the adenylate cyclase responsiveness to calcium is not the site of derangement in these pathological states.

i Studies in collaboration with J. Moss (NHLBI) have identified a heat stable factor from parathyroid cells which activates a purified (calmodulin depleted) preparation of brain phosphodiesterase only in the presence of calcium. This is consistent with findings in numerous (and perhaps all) cell types for a mediator of intracellular calcium, i.e. calmodulin. Trifluoperazine, an antagonist of the active calcium-bound form of calmodulin, inhibits PTH secretion in the presence of low or high extracellular calcium. Thus, although agents which lead to increased entry of calcium into parathyroid cells (ionophore A23187) inhibit secretion, some process(es) in the chain of events leading to exocytosis may require calcium. Quantitation (by phosphodiesterase assay) of calmodulin activity showed that cells from either adenomatous or hyperplastic glands contain approximately 4-fold the activity of normal glands. The implications of this finding are not clear.
[Dr. M. Attie, NIAMDD; Dr. J. Moss, NHLBI]

The cooperative study between investigators in NIAMDD and members of the staff of Hadassah Medical School-Hebrew University involving approximately 300 ambulatory patients with osteoporosis in Jerusalem is now in its seventh year and nearing completion. Accession of new patients has been stopped. By a combination of dietary survey, X-ray, bone mineral content, medical history and other methods, considerable information on the natural history of osteoporosis is anticipated; in addition, a double-blind study is being made of the possible value of oral administration of high calcium, high phosphate and fluoride in treatment of osteoporosis. At the dosage of fluoride being used, 20 mg F per day, undesirable side effects have been very low. Although the number of patients either medically unfit for the study or unwilling to cooperate has been much higher than expected, once entered into active study the drop-out rate has been moderate. This study is also in the process of adding certain new techniques for evaluation of physiologic factors affecting bone metabolism: intestinal absorption tests (^{47}Ca), blood levels of a variety of vitamin D metabolites and fluoride tolerance tests. In addition, in association with this project members of the Physics Department, Hadassah Medical Organization, are being supported in the development of a relatively new technique of bone densitometry, Compton scattering, which it is hoped may be applied to the spine, a most difficult location in which to make such measurements, yet the most critical for assessment of the status of osteoporosis. [Dr. P. H. Bennett, Dr. G. D. Whedon]

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 43001-15 MD

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies in Bone Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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G. D. Whedon, M.D., Director, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Senior Investigator, MDB, NIAMDD
J. Shapiro, M.D., Associate Director, Clinical Center, NIH.

COOPERATING UNITS (if any)

Osteoporosis and Fracture Research Institute, Hadassah Medical Organization, Jerusalem, Israel.

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.25	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of the project on bone metabolism are to investigate the factors influencing mineral storage and loss in demineralizing bone diseases and to study the influence of added calcium and phosphate on resorption of bone mineral. The project involves estimation of bone density by photon absorptiometry and various techniques relative to vitamin D and fluoride metabolism.

During the past year, clinical studies of osteoporosis have been continued, using photon absorption densitometry, with observations of the bone mineral content in various populations and of the possible effect thereon of dietary manipulation.

PHS-6040

Objectives: To investigate the factors influencing mineral storage and loss in demineralizing bone diseases.

Methods Employed: (1) Estimation of bone density in the radius by photon absorptiometry. (2) Estimation of intestinal absorption of calcium by oral administration of tracer doses of ^{47}Ca. (3) Tests of fluoride tolerance.

Major Findings: Studies have continued in order to evaluate the clinical usefulness of photon absorption method of bone mineral analysis. Using this technique, with an iodine-125 source, values for bone mineral content in a normal population of children and adults were determined. These studies demonstrated a fairly linear increase in bone mineral content from birth into the third decade; thereafter bone mineral decreased in both men and women, but at a faster rate in women than in men.

Measurement of bone mineral content in the radius in black populations from birth through adult life disclosed a greater mineral content in blacks compared with whites at all ages. Based on group data (single or few measurements each on many individuals), black females lose mineral in the radius with aging. These studies confirm the clinical impression that osteoporosis is very uncommon in blacks and suggests that as currently defined osteoporosis should not occur as a primary disease in black males. Bone mineral content was determined in black patients with sickle cell anemia. Sixty-four percent of males and 39 percent of females with sickle cell disease have less bone mineral than normal black children of the same age. The ratio of bone mineral content to bone width followed the normal pattern in these children. Loss of bone mineral content did not correlate with severity of anemia or transfusion requirement.

The cooperative study between investigators in NIAMDD and members of the staff of Hadassah Medical School-Hebrew University involving approximately 300 ambulatory patients with osteoporosis in Jerusalem is now in its seventh year and nearing completion. Accession of new patients has been stopped. By a combination of dietary survey, x-ray, bone mineral content, medical history and other methods, considerable information on the natural history of osteoporosis is anticipated; in addition, a double-blind study is being made of the possible value of oral administration of high calcium, high phosphate and fluoride in treatment of osteoporosis. At the dosage of fluoride being used, 20 mg F per day, undesirable side effects have been very low. Although the number of patients either medically unfit for the study or unwilling to cooperate has been much higher than expected, once entered into active study the drop-out rate has been moderate. This study is also in the process of adding certain new techniques for evaluation of physiologic factors affecting bone metabolism: intestinal absorption tests (^{47}Ca), blood levels of a variety of vitamin D metabolites and fluoride tolerance tests. In addition, in association with this project members of the Physics Department, Hadassah Medical Organization, are being supported in the development of a relatively new technique of bone densitometry, Compton scattering, which it is hoped may be applied to the spine, a most difficult location in which to make such measurements, yet the most critical for assessment of the status of osteoporosis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43002-15 MD

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Structure, Secretion and Mechanism of Action of Parathyroid Hormone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, MDB, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Senior Investigator, MDB, NIAMDD
R. W. Downs, Jr., M.D., Senior Clinical Associate, MDB, NIAMDD
M. F. Attie, M.D., Clinical Associate, MDB, NIAMDD
M. A. Levine, M.D., Clinical Associate, MDB, NIAMDD
R. Lasker, M.D., Clinical Associate, MDB, NIAMDD
M. F. Brennan, M.D., Surgery Branch, National Cancer Institute
J. T. Potts, Jr., M.D., Massachusetts General Hospital, Boston
H. T. Keutmann, M.D. "
M. Rosenblatt, M.D. "
T. Murray, M.D., Visiting Scientist, MDB, NIAMDD
R. Rizzoli, M.D., Visiting Scientist, MDB, NIAMDD

COOPERATING UNITS (if any)

Endocrine Unit, Massachusetts General Hospital

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.50	1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Little is known about the nature of circulating parathyroid hormone in clinical disease. It is the purpose of this project to study the function and mechanism of action of parathyroid hormone, study its relationship to human disease, and develop clinically useful tests for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine, porcine and human parathyroid hormone have been determined. Synthetic polypeptides representing the first 34 residues of bovine and human parathyroid hormone have been prepared. These molecules show all the biological properties of the native hormonal polypeptide. A highly sensitive radioimmunoassay for the hormone has been developed and is being modified further for potential use as a specific clinical tool. Studies show that the mechanism of action of the hormone is mediated through direct hormonal activation of adenylate cyclase in bone and kidney. An isolated parathyroid cell system has been developed that allows studies of secretagogues for parathyroid hormone.

Objectives: To study chemical properties and structure of parathyroid hormone; to relate chemical properties and structure to biological activity and to study the mechanism of action of the hormone; to develop valid radioreceptor assays for the hormone; to develop clinically useful tests for circulating parathyroid hormone; to study the physiological regulation of secretion of the hormone.

Methods employed: The hormone is assayed in vivo in rats, in vitro by measuring hormonal activation of adenylate cyclase, or by a highly sensitive radioimmunoassay. Adenylate cyclase is measured by determining conversion of α-^{32}P-labeled ATP to cyclic 3',5'-AMP. The hormone is iodinated by electrolytic or enzymatic methods. Endogenous concentration of cyclic 3',5'-AMP or cyclic 3',5'GMP is determined by radioimmunoassay.

Major findings: mechanism of action. Our earlier studies showed that the parathyroid status of animals is an important factor regulating excretion of cyclic AMP in the urine. This finding led to development of the thesis that cyclic AMP is involved in the mediation of parathyroid hormone action. Subsequent work showed that parathyroid hormone in vitro activates adenylate cyclase in the kidney as well as in bone, the two organs known to be physiological receptors for action of the hormone. Adenylate cyclase in the kidney is found in two different anatomic zones; adenylate cyclase in the cortex responds specifically to parathyroid hormone whereas that in the medulla responds to vasopressin. This work and numerous corroborative studies in other laboratories established that the receptor for parathyroid hormone in kidney resides within the renal cortical tubule as had been suspected but not proven from indirect in vivo physiological experiments. Recent studies suggest that it is possible to iodinate parathyroid hormone electrolytically to provide a useful ligand for use in radioreceptor systems (Drs. Murray, Rizzoli and Aurbach).

Human parathyroid hormone. Parathyroid hormone has been purified from human parathyroid adenomas and the complete structure of the molecule (Drs. Potts and Rosenblatt) has been determined. Based on this analysis, Drs. Potts and Rosenblatt (Boston) have synthesized the tetratriacontapeptide representing the amino terminus of the molecule. The product is fully active in vitro and in vivo and studies have already been reported on clinical use of the peptide. [Dr. G.D. Aurbach; Drs. Potts, Rosenblatt and Keutmann]

Studies have continued with dispersed bovine and human parathyroid cells. PTH secretion from parathyroid glands in vivo and cells in vitro is inhibited by calcium. Calcium may also modulate synthesis and degradation of PTH. This control exerted by calcium may be changed in certain pathologic states (glandular adenomas, carcinomas and perhaps hyperplasia). Several earlier studies have strongly suggested that cAMP is an intracellular mediator of PTH secretion; agents which alter cellular cAMP change PTH secretion in the same direction. Calcium decreases cellular cAMP although some of its effects may be independent of cAMP. The adenylate cyclase of the parathyroid cell is a potential regulatory site for the action of calcium. Adenylate cyclase in homogenates of dispersed parathyroid cells shows sensitivities to Ca (1/2 maximal

inhibition at approximately 0.2 mM added calcium) that are similar in human parathyroid adenomas, carcinomas or hyperplastic glands and like that of normal bovine glands. There was no correlation between the in vitro calcium suppressibility of PTH release by dispersed cells (several of the adenomas and the one carcinoma were not suppressed by high extracellular calcium) and the sensitivity of the adenylate cyclase in the broken cell preparation. This suggests that the adenylate cyclase responsiveness to calcium is not the site of derangement in these pathological states.

Studies in collaboration with J. Moss (NHLBI) have identified a heat stable factor from parathyroid cells which activates a purified (calmodulin depleted) preparation of brain phosphodiesterase only in the presence of calcium. This is consistent with findings in numerous (and perhaps all) cell types for a mediator of intracellular calcium, i.e. calmodulin. Trifluoperazine, an antagonist of the active calcium-bound form of calmodulin, inhibits PTH secretion in the presence of low or high extracellular calcium. Thus, although agents which lead to increased entry of calcium into parathyroid cells (ionophore A23187) inhibit secretion, some process(es) in the chain of events leading to exocytosis may require calcium. Quantitation (by phosphodiesterase assay) of calmodulin activity showed that cells from either adenomatous or hyperplastic glands contain approximately 4-fold the activity of normal glands. The implications of this finding are not clear.
[Dr. M. Attie, NIAMDD; Dr. J. Moss, NHLBI]

Significance to Biomedical Research and Program of the Institute: Parathyroid hormone controls the biology of skeletal tissue, mineral metabolism and renal function. These control mechanisms are directly related to programs of the Institute on renal disease, hyperparathyroidism, vitamin D metabolism, osteoporosis, and other metabolic bone diseases. This project since its inception has yielded significant new information and insights relative to these Institute programs. The discovery that cyclic AMP is the mediator of parathyroid hormone action led to the elucidation of cyclic AMP as an important controlling biochemical intermediate in bone resorption mediated by a variety of biologically important factors. This discovery similarly led to important new clinical laboratory tests for hyperparathyroidism, pseudohypoparathyroidism and other disorders of parathyroid secretion and function.

Proposed Course of Project: Studies with isolated parathyroid cells will continue in a search for factors that control parathyroid hormone secretion. The mechanism whereby calcium controls cell function will also be examined with the isolated parathyroid cell preparation. Studies will continue in attempts to delineate the nature of circulating parathyroid hormone. Methods for identifying parathyroid hormone receptors in target tissues will continue to be explored. Intense interest persists in the application of knowledge from these projects to problems of clinical disease.

Publications:

1. Aurbach, G. D., and Phang, J. M.: Vitamin D, parathyroid hormone, and calcitonin. In Mountcastle, V. B. (Ed.): Medical Physiology, 14th Edition. Saint Louis, The C. V. Mosby Co., 1980, pp. 1519-1557.

2. Gardner, D. G., Brown, E. M., and Aurbach, G. D.: Inhibition of adenosine 3',5'-monophosphate accumulation and parathyroid hormone release by sodium nitroprusside. Endocrinology 105: 360-366, 1979.

3. Brown, E. M., Gardner, D. G., and Aurbach, G.D.: Effects of calcium ionophore A23187 on dispersed bovine parathyroid cells. Endocrinology 106: 133-138, 1980.

4. Gardner, D. G., Brown, E. M., Attie, M. F., and Aurbach, G. D.: Prostaglandin-mediated stimulation of adenosine 3',5'-monophosphate accumulation and parathyroid hormone release in dispersed human parathyroid cells. J. Clin. Endocrinol. Metab. 51: 20-25, 1980.

5. Aurbach, G. D., and Brown, E. M.: Secretory mechanisms and actions of parathyroid hormone and calcitonin. In Munson, P. L., Diczfalusy, E., Glover, J., and Olson, R. E. (Eds.): Vitamins and Hormones. New York, Academic Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 43003-14 MD

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies on the Mode of Action of Thyrocalcitonin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.50	.25	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose is to study the interaction of calcitonin with its specific receptor target organs. The current investigations should provide further insight into the structure function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful for characterizing hormone receptors in kidney, bone and other tissues. Studies are in progress to characterize further the interaction of calcitonin with tissue receptors. It also will be of interest to solubilize the receptors and characterize them chemically.

Objectives: To study the interaction of calcitonin with its specific receptor target organs.

Methods Employed: The interaction of calcitonin with its receptors has been evaluated by determining adenylate cyclase as well as binding of radioiodinated calcitonin to membranes isolated from renal tissue and to lymphoid cells isolated from human and other species or grown in tissue culture.

Major Findings: A purification procedure for kidney membranes has been developed. It has been shown that the calcitonin receptor from the kidney is concentrated at the corticomedullary junction. The membranes which bind calcitonin and contain calcitonin-sensitive adenylate cyclase show low specific activities for renal brush border enzymes, indicating that calcitonin receptors are localized at an anti-luminal aspect of renal cells or at a site that does not contain brush borders. Utilizing a series of calcitonin analogs, dose response curves were compared for binding to receptors from kidney and bone. The dose response curves in the two tissues are virtually superimposable, suggesting that the calcitonin receptor in the two organs has a similar molecular structure. The interaction of the hormone-receptor complex with the adenylate cyclase enzyme is modified by the presence of guanine nucleotides. In the presence of GMPPNP the threshold for hormonal activation is lowered.

The relative renal responses to calcitonin analogs are similar whether determined by competitive binding or adenylate cyclase activation. Thus potency differences of the analogs tested are largely dependent upon effectiveness at the receptor level. Activity of the adenylate cyclase enzyme of human lymphocytes was not modulated by ambient or bound calcitonin.

Calcitonin receptors were identified on cultured human lymphocytes. The characteristics of these receptors are similar to those on rat renal and calvarial membranes as determined by competitive binding studies. The lymphocyte receptors, however, do not appear capable of modulating adenylate cyclase enzyme in that cyclic AMP was not detected after adding calcitonin to intact cells. Lymphocyte preparations should prove useful, however, in that they represent a readily obtainable human tissue containing calcitonin receptors. Results with this human material indicated that, although calcitonin polypeptides have undergone remarkable evolutionary changes in structure and effectiveness, there has been no significant corresponding change in biological nature of calcitonin receptors.

Significance to Biomedical Research and Program of the Institute: The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful characterizing hormone receptors present in kidney, bone, and other tissues.

Proposed Course of Project: Further studies are in progress to characterize further the interaction of calcitonin with its tissue receptors. It also will be of interest to solubilize calcitonin receptors and further characterize them. Purified lymphoid cell types are being characterized with respect to the density of calcitonin receptors on their surface. The whole cell system also may be applied to evaluate the regulation of receptor metabolism in vitro and in human disease states.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 43004-15 MD

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies on pseudohypoparathyroidism and related disorders

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Senior Investigator, MDB, NIAMDD
R. W. Downs, Jr., M.D., Senior Clinical Associate, MDB, NIAMDD
M. A. Levine, M.D., Clinical Associate, MDB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	.75	.50

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. We previously found that patients with PHP lack the normal rise in U_{cAMP} excretion seen following PTH administration. This indicated that ~~there is~~ a defective hormone receptor-adenylate cyclase complex in this disorder. We have now shown that red cells from patients with PHP are functionally deficient in a recently isolated protein ("G unit") that serves to couple hormone receptor to the catalytic unit of adenylate cyclase. Our results suggest that reduction in G units is the lesion responsible for hormone resistance in PHP and imply that the defect is not limited to PTH target organs (kidney and bone).

PHS 6040

Project Description. In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Albright proposed that this disorder was attributable to lack of end organ sensitivity to normally-secreted endogenous parathyroid hormone. Our previous finding that patients with PHP lack the expected rise in U_{cAMP} excretion following PTH administration implicated a defective hormone receptor-adenylate cyclase complex as the basis for the disorder. Current studies have taken advantage of recent advances in understanding of the components of the receptor-cyclase complex and have attempted to pinpoint the component of the complex responsible for the clinically observed hormone resistance.

Methods: Red cells were obtained by venipuncture and plasma membranes prepared by hypotonic lysis. The G unit was extracted from red cell membranes with nonionic detergents. G unit was assayed by measuring the adenylate cyclase activity generated by combining human red cell membrane extracts (source of G unit) with one of two sources of catalytic units: a) turkey red cell membrane extracts or b) plasma membranes from the CYC^- clone of S49 mouse lymphoma cells (kindly provided by Drs. Nielsen and Rodbell, LNE, NIAMDD).

Major Findings: Red cell membranes from patients with PHP had reduced G unit activity (mean reduction = 50%) by comparison with both normal controls and patients with surgical hypoparathyroidism. The reduction in G unit activity was highly significant ($p < .001$) in both the turkey erythrocyte and CYC^- membrane assay systems. We found no alteration in affinity for guanine nucleotide of the G unit from red cells of patients with PHP. Only 1 of 15 patients with "classic" (somatic features and PTH resistance) PHP showed normal red cell G unit activity.

The mode of inheritance of this disorder remains unclear but the finding of ~50% reduction in G unit activity and the preponderance of affected females is compatible with sex-linked dominant inheritance. Genetic heterogeneity is suggested by the finding of one patient (a male) in our study group with normal red cell G unit activity, as well as by the occurrence of PTH resistance in individuals lacking the somatic features described by Albright (red cell G unit activity was normal in one patient studied by us with this form of the disorder).

Our findings suggest that reduction in functional G units is the molecular basis for hormone resistance in most patients with PHP. Since the G unit, unlike receptors for a given hormone, e.g. PTH, is not tissue-specific, we would anticipate that all tissues manifest the lesion. This hypothesis, bolstered by our findings in red cell membranes, may explain why patients with PHP often show resistance to other hormones whose action is mediated by cAMP, e.g. TSH.

Significance to Biomedical Research and Program of the Institute: Our findings represent an important link between basic studies on the structure and function of the hormone receptor-adenylate cyclase complex and clinical studies of various endocrine disorders. While PHP itself is a rare disease, it provides an important model for the study of normal and abnormal hormone action. Our studies suggest that PHP may represent the first example of a human disease involving a defect in the G-unit protein only recently isolated and characterized. As such, PHP provides an important opportunity to assess the functional significance of this protein, the G unit in humans. Better understanding of the function of the G unit should increase our ability to define the pathophysiology of endocrine hyper- and hypofunctional states and may provide novel possibilities for therapeutic intervention.

Proposed Course of Project: The immediate goals of our project are 1) to verify the reduction of functional G units in tissue other than red cells from patients with PHP, 2) to attempt to elucidate the nature of the defect in patients with PHP who show normal G unit activity, and 3) to study the responsiveness of a variety of organs in patients with PHP to trophic hormones whose action is mediated by cAMP.

Publication

Levine, M. A., Downs, R. W., Jr., Singer, M. J., Jr., Marx, S. J., Aurbach, G. D., and Spiegel, A. M.: Deficient activity of guanine nucleotide regulatory protein in erythrocytes from patients with pseudohypoparathyroidism. Biochem. Biophys. Res. Commun. 94: 1319-1324, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER A01 AM 43005-15 MD

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Hormone Receptors, Adenylate Cyclase and Cyclic AMP-Mediated Ion Transport

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
A. M. Spiegel, M.D., Senior Investigator, MDB, NIAMDD
R. W. Downs, Jr., M.D., Senior Clinical Associate, MDB, NIAMDD
M. F. Attie, M.D., Clinical Associate, MDB, NIAMDD
M. A. Levine, M.D., Clinical Associate, MDB, NIAMDD
W. Krawietz, M.D., Guest Worker, MDB, NIAMDD
J. D. Gardner, M.D., Chief, Digestive Diseases Branch, NIAMDD
T. Nielsen, M.D., Laboratory of Nutrition and Endocrinology, NIAMDD
M. Rodbell, M.D., LNE, NIAMDD

COOPERATING UNITS (if any)
Section on Gastroenterology, Digestive Diseases Branch, NIAMDD
Laboratory of Nutrition and Endocrinology, NIAMDD

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.25	2.50	2.75

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied the components of the hormone receptor-adenylate cyclase complex. The turkey erythrocyte, a readily available, homogeneous cell type has been used as a model system. The turkey RBC plasma membrane possesses β-adrenergic receptors, identified using radioiodinated hydroxybenzylpindolol, a high-affinity β-blocker. β-receptors are coupled to adenylate cyclase catalytic units by a guanine nucleotide binding protein (G-unit) which we have isolated by affinity chromatography. We have studied the role of the G unit in mediating activation of adenylate cyclase by hormone, fluoride and cholera toxin. β-adrenergic agonist stimulation of cAMP accumulation in turkey erythrocytes leads to significant changes in sodium and potassium transport.

Objectives: To isolate and purify the components of the hormone receptor-adenylate cyclase complex from particular tissues, to determine the nature of the interaction of the various components, and to determine the nature of the link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems.

Methods Employed: Polypeptide and catecholamine hormones are purified and iodinated by standard methods or methods developed in the laboratory specifically for that purpose. Rate of association of hormones is determined by incubating plasma membrane fractions or whole cell with labeled hormones. The kinetics of binding is determined by rate measurements as well as assessment of steady state conditions. Specific hormone binding is defined in terms of dissociation constant (K_m) and the physiological characteristics of the system.

Adenylate cyclase is assayed in intact plasma membranes or in detergent extracts (Lubrol PX) by monitoring conversion of [α-^{32}P]ATP to [^{32}P]cAMP. The G unit is assayed by combining it with catalytic units from turkey erythrocyte or CYC$^-$ clone of S49 mouse lymphoma cell plasma membranes (supplied by T. Nielsen and M. Rodbell, LNE, NIAMDD) and measuring the resultant increase in adenylate cyclase activity. The G unit is isolated from detergent extracts of turkey erythrocyte membranes by affinity chromatography on a GTP (γphosphate linked) matrix. Cholera toxin and [^{32}P]NAD are used to radiolabel covalently the G unit in various plasma membranes.

Major Findings: The G unit has been partially purified from turkey erythrocyte membranes with GTP affinity chromatography. Our studies show that the G unit is reversibly associated with the catalytic unit of the solubilized holoenzyme. The partially purified G unit from turkey erythrocyte restores both guanine nucleotide and fluoride responsive adenylate cyclase activity to homologous (turkey erythrocyte) and heterologous (CYC$^-$ S49 mouse lymphoma cell line genetically deficient in G units) catalytic units.

A method has been developed to radiolabel covalently the G unit in turkey erythrocyte membranes. Cholera toxin catalyzes the transfer of [^{32}P]NAD to a subunit of the regulatory protein which has an apparent molecular weight of 42,000 on SDS gel electrophoresis. Labeling of the regulatory protein is cholera toxin specific, and is distinct from nonspecific labeling of other proteins which occurs without toxin. [^{32}P]ADP-ribosylation permits detection of the G unit during detergent solubilization, affinity purification, sucrose gradient centrifugation, and other manipulations. The radiolabeled 42,000 M_r peptide and the G unit (as measured by reconstitution of adenylate cyclase activity) coelute from the GTP affinity column upon addition of specific guanine nucleotides. Cholera toxin-mediated ADP ribosylation of the G unit alters turkey erythrocyte adenylate cyclase activity so that GTP becomes almost as effective as its non-hydrolyzable analogs, e.g. GTP-γ-S. A 42,000 M_r protein is also radiolabeled with cholera toxin and [^{32}P]NAD in human erythrocyte membranes. This correlates with the presence of functional G units in human red cells as measured by G unit assay.

Significance to Biomedical Research and Program of the Institute: Studies of the structure and function of the components of the hormone receptor-adenylate cyclase complex increase our understanding of the mechanisms of hormone action. Recognition of the critical role of the G unit as a "coupling factor" in transmitting hormonal messages across cell membranes makes it likely that various disease states may involve G unit malfunction. Studies in our laboratory on the hormone resistance syndrome, pseudohypoparathyroidism, bear out this possibility (see separate PHP poject report). G unit alterations may also lead to increased adenylate cyclase activity. This is best exemplified by the effects of cholera toxin. Indeed the severe secretory diarrhea seen in clinical vibrio cholera infection results from G unit ADP-ribosylation and consequent increased cAMP production in the gut. Further studies on the structure and function of the G unit may open up new possibiliies for therapeutic intervention in established cases of cholera. Labeling of the G unit with cholera toxin and [^{32}P]NAD may permit detection of quantitative or qualitative alterations in the G unit in various human disease states.

Proposed Course of Project: Future goals of this project include further purification of the G unit, production of antibodies to the G unit to aid in detection of genetic abnormalities in G unit function, study of the mechanism of coupling between the G unit and both hormone receptors and catalytic unit, and study of the relationship between the G unit and effects of guanine nucleotides on agonists (e.g. α-adrenergic, opiates, muscarinic) that act via inhibition of adenylate cyclase.

Publications

Downs, R. W., Jr., Spiegel, A. M., Singer, M., and Aurbach, G. D.: Fluoride stimulation of adenylate cyclase is guanine nucleotide dependent. J. Biol. Chem. 255: 949-954.

Aurbach, G. D.: Receptor-adenylate cyclase components: abnormalities in clinical medicine. In Hamet, P. and Sands, H. (Eds.): Advances in Cyclic Nucleotide Research. New York, Raven Press, 1980, in press.

Aurbach, G. D.: Book review (Advances in Cyclic Nucleotide Research, Volume 10; Brooker, G. and Greengard, P., editors; Raven Press, New York, 1980). Anal. Biochem. 101: 514-515, 1980.

Nielsen, T. B., Downs, R. W., Jr., and Spiegel, A. M.: Restoration of guanine nucleotide and fluoride stimulated activity to an adenylate cyclase deficient cell line with affinity purified guanine nucleotide regulatory protein. Biochem. J., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER A01 AM 43006-05 MD

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Study of Hyperparathyroidism: Etiology, diagnosis and treatment.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
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LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.00	1.50

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project goal is the evaluation and treatment of hyperparathyroidism. Patients with persistent postoperative hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the multiple endocrine neoplasia syndromes. Evaluation ranges from epidemiologic studies of families to in-house studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include radioimmunoassay of parathyroid hormone, selective arteriography and selective venous sampling for parathyroid hormone, parathyroid gland cryopreservation and autotransplantation, and transcatheter parathyroid gland infarction.

PHS-6040

Objectives: To study the regulation of parathyroid gland function. To develop and evaluate diagnostic tests of parathyroid function. To develop new methods for localization of hyperfunctioning parathyroid tissue. To evaluate methods of treatment of hyperparathyroidism.

Methods employed: Patients with suspected hyperparathyroidism are referred for evaluation and treatment. Evaluation includes radioimmunoassay of circulating parathyroid hormone, and of plasma and urinary cyclic AMP. Where appropriate, preoperative evaluation includes selective arteriography and venography with selective sampling for PTH determination and computerized tomography of neck and mediastinum. In selected cases adenomatous parathyroid glands are ablated by transarterial infusion of various agents. Parathyroid exploration is done by the Surgery Branch, NCI. Urinary cyclic AMP (U_{cAMP}) excretion is monitored during surgery, utilizing the gammaflo machine for rapid (<7 min) radioimmunoassay of cyclic AMP. If postoperative hypoparathyroidism is considered highly likely, parathyroid tissue may be simultaneously autotransplanted to a forearm muscle. Excised parathyroid tissue either fresh or cryopreserved is evaluated in vitro after preparation of dispersed cells. If postoperative hypoparathyroidism is considered possible, tissue is cryopreserved for subsequent autotransplantation. Techniques for cryopreserving and grafting parathyroid tissue are being studied in an experimental model with adult dogs.

Major findings: Diagnosis and clinical findings: A large number of patients with primary hyperparathyroidism have been treated and their clinical and chemical findings have been described. Weakness was noted more commonly than had previously been appreciated and muscle biopsy showed atrophy of type I and especially type II fibers in a manner diagnostic of denervating disease. Studies of neuromuscular function in subjects with secondary hyperparathyroidism showed similar clinical and histologic findings. The most useful diagnostic tests for hyperparathyroidism have been hypercalcemia associated with nonsuppressed or increased parathyroid hormone concentrations in peripheral blood and increased urinary excretion of cyclic AMP. These findings revert rapidly to normal following successful parathyroidectomy. Intestinal absorption of calcium is increased preoperatively and undoubtedly has a central role in causing hypercalcemia.
[G.D.Aurbach, S.J.Marx, A.M.Spiegel, R.W. Downs, J.L. Stock, M.A. Levine, R. Lasker, MDB, NIAMDD: A. Saxe and M. Brennan, Surgery Branch, NCI]

Although results with parathyroid radionuclide imaging after intravenously administered [^{75}Se]-selenomethionine have been disappointing, we have been obtaining parathyroid scans after administering this isotope intra-arterially during parathyroid arteriography. Several cases wherein cannulation of most of the important vessels potentially supplying the parathyroids was possible have shown correctly localizing scans although careful interpretations are necessary to avoid false positives. This technique appears to be especially useful in those patients with juxta- or intra-thyroidal adenomas even when arteriography is ineffective. Radionuclide images are often positive because suppression of the thyroid with triiodothyronine prior to scanning improves the specificity of the parathyroid scan but not of the arteriogram.
[Drs. Marx, Spiegel, Downs, Stock and Aurbach, NIAMDD; Drs. Doppman, Jones and Krudy, Radiology Department, C.C.]

We have studied a patient with parathyroid carcinoma who showed markedly elevated serum concentrations of the glycoprotein hormone subunits hCG-α and hCG-β, which fell dramatically after surgical removal of malignant tissue. Screening of a large number of patients with different forms of hyperparathyroidism has shown these subunits to be a marker for malignancy in parathyroid disease or for islet cell involvement in familial multiple endocrine adenomatosis (MEN I).
[Drs. Marx, Spiegel, Downs, Stock and Aurbach, MDB, NIAMDD; Drs. Weintraub and Rosen, CEB, NIAMDD]

Several studies have utilized urinary cyclic AMP excretion as a parameter of parathyroid hormone action:

Our previous studies showed that intraoperative U_{cAMP} excretion fell rapidly to the normal range (mean = 1.5 hrs) after successful parathyroidectomy but failed to fall after unsuccessful surgery in patients with primary hyperparathyroidism (1°HPT). Availability of the gammaflo machine (capable of performing cAMP radioimmunoassay in <7 min/sample) enabled us to study prospectively the utility of rapid intraoperative U_{cAMP} determination in guiding surgery for 1°HPT. The data from over 20 patients studied with this technique to date confirm the specificity of intraoperative U_{cAMP} excretion as an index of parathyroid gland function. Rapid U_{cAMP} determination proved to be extremely useful in several patients. A fall in U_{cAMP} to the normal range obviated the need for further neck exploration (with attendant increased morbidity) even when histologic confirmation of removal of abnormal parathyroid glands was lacking. Persistent (>1.5 hrs) elevation of U_{cAMP} signaled the need to continue exploration even after one or more enlarged glands had been removed. A peak in U_{cAMP} excretion above baseline was a clue to the location of an enlarged gland not found during previou exploration. This technique appears to be a valuable tool in assisting the surgeon exploring the particularly difficult group of patients who have undergone prior unsuccessful neck surgery.

Familial hyperparathyroidism. Surveys of families of patients with primary parathyroid hyperplasia show that hereditary disease is very common (approximately 50% in our series of 25 index cases with primary parathyroid hyperplasia). In two large kindreds a novel form of familial hypercalcemia was found wherein hypercalcemia occurs with hypocalciuria (FHH). Unlike typical cases of familial hyperparathyroidism, the penetrance of hypercalcemia approached 100% in the first two decades with severe expression even in one neonate. Review of results of surgery at the N.I.H. and elsewhere has shown that all of 6 patients in this category treated by subtotal parathyroidectomy had persistent postoperative hypercalcemia. While elevated concentrations of glucagon and/or gastrin were common in subjects with familial multiple endocrine neoplasia type I, these were not elevated in hypercalcemic members of these two families. In these families renal clearances of filtered calcium and magnesium are both lower than in typical primary hyperparathyroidism and circulating parathyroid hormone levels are lower than in typical primary hyperparathyroidism. Circulating parathyroid hormone concentrations, assessed by three independent assay techniques, are lower in these patients than in patients with typical primary hyperparathyroidism. Furthermore, the cAMP response to infused PTH is exaggerated

in these patients. In a series of 67 patients referred after exploration for hypercalcemia, six (9%) were previously unrecognized probands for kindreds with FHH. Analysis of urine calcium expressed as ratio of calcium clearance:creatinine clearance facilitates recognition of this disorder. Recognition of FHH in the population is important in order to obviate unnecessary parathyroid surgery in these cases. [S.J.Marx, A.M.Spiegel, R.W.Downs,Jr., M.Attie, M.A.Levine, R.Lasker, and G.D.Aurbach, MDB,NIAMDD; J.Gardner, DDB, NIAMDD] A

Application of new radiologic techniques: A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization of parathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral vein and thence through the major veins to reach the neck where selective samples are taken from each inferior and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10- to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to samples from the general circulation. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by nonparathyroid cancers.

Thyroid and internal mammary arteriography enables preoperative identification of hyperfunctioning parathyroid tissue in 30% or more of cases and is also helpful in defining venous drainage to facilitate selective catheterization. In eight cases of mediastinal parathyroid adenoma treatment was accomplished by transarterial infusion of hypertonic glucose, gelfoam or autologous clot to occlude the catheterized parathyroid artery. Seven appear fully cured and one partially. There was no significant morbidity to these patients who would otherwise have required sternotomy. [G.D.Aurbach, S.J.Marx, A.M.Spiegel, R.W. Downs, Jr., and J.L. Stock, MDB, NIAMDD; A. Krudy and J. Doppman, Radiology Dept., C.C., NIH]

Autotransplantation and cryopreservation of parathyroid: Persistent postoperative hyperparathyroidism or hypoparathyroidism are potential problems in patients with primary parathyroid hyperplasia or previous unsuccessful parathyroid exploration. These problems may be lessened if parathyroid tissue can be cryopreserved. We have developed experience with cryopreservation and autotransplantation of parathyroid glands in normal dogs and also in a few patients who developed permanent hypoparathyroidis postoperatively. Avoidance of tissue trauma or rapid changes in temperature or pH was essential for tissue preservation. Canine parathyroid tissue has been successfully autotransplanted after cryopreservation for as long

as nine months and function has been documented by measuring release of immunoreactive PTH and by induction of hypocalcemia following graft excision. Function of autografted parathyroid tissue in vivo correlates well with assessment in vitro. One patient who received an autograft from a parathyroid adenoma developed rapid recurrence of primary hyperparathyroidism. Subsequently his tissue showed highly abnormal function in vitro and in vivo. Graft excision was required and he became hyoparathyroid.
[G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, R.W.Downs,Jr., M.A.Levine, and R.Lasker, MDB,NIAMDD; A. Saxe and M. Brennan, Surgery Branch, NCI]

Significance to Biomedical Research and Program of the Institute: As an outgrowth of these studies, methods of localizing and treating hypersecreting tissues of several types are being improved. Hyperparathyroidism is a prototype of endocrine hyperfunction. Some patients have hyperparathyroidism in combination with hereditary hyperfunction of other endocrine glands including pituitary and pancreas. These hyperfunctions are usually benign but occasionally become cancerous. By studying families with hereditary disease we hope to gain insight into specific molecular lesions that cause loss of normal cellular regulation in these tissues. This collaborative program offers comprehensive treatment for a select group of patients who have proven difficult or impossible to treat in other centers in the United States and abroad.

Proposed Course of Project: Patients with suspected primary hyperparathyroidism will be accepted for evaluation and treatment. We will continue to study familial patterns of hyperparathyroidism and efforts will continue towards improving techniques for assessment of parathyroid hormone by radioimmunoassay or bioassay. We will continue to explore new methods of localizing hyperfunctioning parathyroid tissue. The applicability of parathyroid embolization and the role of parathyroid autotransplantation will be investigated further. Patients in the entire program will provide a source of abnormal parathyroid glands to be studied in detail in vitro. Patients with parathyroid transplants will be ideal test subjects for putative parathyroid secretagogues.

Publications

1. Marx, S. J., Spiegel, A. M., Brown, E. M., Gardner, D. G., Downs, R. W., Jr., Atti, M.,and Aurbach, G. D.: Familial hypocalciuric hypercalcemia. In Anast, C. (Ed.): Pediatric Diseases Related to Calcium. New York, Elsevier North Holland, Inc., 1980, pp. 413-431.

2. Aurbach, G. D., Marx, S. J., and Spiegel, A. M.: Parathyroid hormone, calcitonin, and the calciferols. In Williams, R. R. (Ed.): Textbook of Endocrinology, Sixth Edition. Philadelphia, W. B. Saunders Company, in press.

3. Marx, S. J., Stock, J. L., Attie, M. F., Downs, R. W., Jr., Gardner, D. G., Brown, E. M., Spiegel, A. M., Doppman, J. L., and Brennan, M. F.: Familial hypocalciuric hypercalcemia: recognition among patients referred after unsuccessful parathyroid exploration. Ann. Int. Med. 92: 351-356, 1980.

4. Marx, S. J., Spiegel, A. M., Sharp, M. E., Brown, E. M., Gardner, D. G., Downs, R. W., Jr., Attie, M. F., and Stock, J. L.: Adenosine 3',5'-monophosphate response to parathyroid hormone: familial hypocalciuric hypercalcemia versus typical primary hyperparathyroidism. J. Clin. Endocrinol. Metab. 50: 546-549, 1980.

5. Thorgeirsson, U., Costa, J., and Marx, S. J.: The parathyroid glands in familial hypocalciuric hypercalcemia. Human Pathology, 1980, in press.

6. McCarthy, D. M., Peikin, S. R., Lopatin, R. N., Long, B. W., Spiegel, A., Marx, S., and Brennan, M.: Hyperparathyroidism - a reversible cause of cimetidine-resistant gastric hypersecretion. Brit. Med. J. 1: 1765-1766, 1979.

7. Aurbach, G. D.: Parathyroid. In Beeson, P.B., McDermott, W., and Wyngaarden, J.B. (Eds.): Cecil Textbook of Medicine, Fifteenth edition. Philadelphia, W. B. Saunders Co., 1979, pp. 2213-2224.

Annual Report of the Digestive Diseases Branch

SUMMARY OF BRANCH ACTIVITIES

The Digestive Diseases Branch has two sections (Section on Gastroenterology and Section on Diseases of the Liver). The Section on Diseases of the Liver has two senior physicians and 3 clinical associates; the Section on Gastroenterology has three senior physicians and 4 clinical associates. The Digestive Diseases Branch also has 5-10 guest investigators from other laboratories.

Detailed summaries of the activities of each section precedes the individual project reports. Both sections are engaged in investigations of basic biologic processes (e.g. hormone action, membrane transport, cellular and humoral immunology) and are attempting to apply this information to understand the pathophysiology of various disorders involving the liver and gastrointestinal tract. Both sections are also involved in attempts to improve therapy of clinical disorders such as neoplasms associated with overproduction of gastrointestinal hormones and fulminant hepatic failure.

Section on Gastroenterology

During the past year we have extended our studies of the biochemical basis of action of gastrointestinal hormones on their target tissues and have centered our investigations of patients with clinical syndromes caused by tumor production of one or more gastrointestinal hormones.

We have developed homogenous suspensions of viable, hormone-responsive cells from various gastrointestinal tissues and have used these preparations to explore the biochemical basis of action of various hormones and neurotransmitters. During the past year we have developed procedures for radiolabeling the various agents that play major roles in regulating the function of different gastrointestinal tissues and have begun to explore the initial step in the action of these agents on their target tissues - binding to surface membrane receptors on the target cell.

Serendipitously we discussed that $N^6,O^{2'}$-dibutyryl cyclic GMP is a competitive antagonist of the action of cholecystokinin on its target tissues. During this past year we have completed an extensive study of the important structural requirements for a compound to antagonize the interaction of cholecystokinin with its membrane receptors. Based on these studies we have established collaborative relations with two different organic chemistry laboratories and hope to be able to develop a compound that can be used as an antagonist of the action of cholecystokinin in man.

By collaborating with various peptide chemists we have been able to study approximately 50 different fragments and analogues of cholecystokinin, 30 analogues of bombesin and 15 analogues of substance P. These peptides have enabled us to determine the minimal structural requirements for interaction with receptors and the structural requirements for various biologic activities.

We are currently treating approximately 30 patients with Zollinger-Ellison Syndrome with cimetidine. This drug appears to constitute effective therapy in this disorder and obviates the need for total gastrectomy. It has also become clear that most patients with Zollinger-Ellison syndrome will die from effects of the tumor per se not from effects of its secreted products. During this past year we have initiated studies of the effects of autineoplastic chemotherapy in patients with the Zollinger-Ellison syndrome. We have found that in 2 of 5 patients the combination of adriamycin, streptozotocin and 5-fluorouracil caused significant amelioration of effects related to the neoplasm.

A substantial majority of patients with the pancreatic cholera syndrome respond satisfactorily to streptozotocin. In those patients who do not respond, management of the voluminous diarrhea is a major clinical challenge. During this past year we have found that in patients with pancreatic cholera syndrome, lithium carbonate can cause a substantial reduction in stool output presumably because of the abilities of lithium to inhibit the action of vasoactive intestinal peptide (the cause of the syndrome) on adenylate cyclase in small intestinal mucosa. Other agents that offer therapeutic promise are the phenothiazines. Recently it has become clear that calmodulin is involved in intestinal secretory processes and agents that inhibit calmodulin may reduce intestinal secretion in the pancreatic cholera syndrome.

Liver Diseases Section

The Liver Diseases Section is currently conducting seven principal studies:

I. Studies of the Pathogenesis of Acute Hepatic Coma and The Use of Artificial Hepatic Support Devices for its Treatment

A highly reproducible model of fulminant hepatic failure (FHF) using the selective hepatotoxin galactosamine hydrochloride has been developed in an inbred strain of rabbits. This model resembles human FHF in all important respects. To investigate the efficacy of various hemoperfusion adsorbents and schedules in the treatment of the model, an extracorporeal hepatic support system for the removal of potentially encephalopathic substances from the plasma of rabbits has been developed. Certain specific surfaces, if incorporated into the hemoperfusion circuit, have been shown to be bioincompatible in that they can induce a fatal syndrome characterized by activation of the complement system, complement-induced aggregation of polymorphonuclear neutrophils and embolization of the aggregated cells to pulmonary arterioles. While corticosteroids can prevent complement-mediated aggregation of polymorphonuclear neutrophils in vitro, hydrocortisone 150 mg intravenously per kilogram body weight per hour did not prevent the pulmonary sequestration of polymorphonuclear neutrophils in vivo when the blood of rabbits was perfused through an extracorporeal hemoperfusion device containing an implicated foreign surface.

The pattern and amplitude of visual evoked potentials in the central nervous system have been monitored in rabbits via chronically implanted epidural electrodes. Repeated visual stimulation with real time computer-

assisted summation increased the reproducibility of visual evoked potentials by removing background electroencephalographic activity. The recording of visual evoked potentials has been shown to be a reliable quick, non-invasive method of quantifying neurologic changes in hepatic encephalopathy. Furthermore, the pattern of visual evoked potentials associated with acute hepatic coma was found to be similar to that associated with coma induced by certain hypnotic agents, e.g. phenobarbital and benzodiazepines, but not others, e.g. ether. As phenobarbital and benzodiazepines are known to interact with postsynaptic receptors for γ-aminobutyric acid (GABA) in the central nervous system, it seems possible that putative encephalotoxins in hepatic encephalopathy might exert their effects via the GABA inhibitory neurotransmitter system.

To investigate GABA metabolism and the status of the GABA neurotransmitter system in hepatic encephalopathy, a highly sensitive and reproducible competitive radioreceptor assay for GABA has been set up and characterized. Studies involving the application of this assay have shown: (i) A major source of GABA in peripheral blood is the enteric bacterial flora; (ii) Levels of GABA in peripheral blood are increased in acute hepatic coma but not in another type of metabolic encephalopathy; and (iii) The density of receptors for GABA on postsynaptic neural membranes of the brain is increased in acute hepatic coma. These findings suggest that in hepatic coma GABA derived from the gut crosses a porous blood-brain barrier and induces its own receptors in the brain. This hypothesis affords potential explanations of the generalized neural inhibition of hepatic encephalopathy and of the increased sensitivity of the brain to certain hypnotic drugs in hepatic encephalopathy. Relevant to this hypothesis are studies (currently in progress) which are designed to determine the porosity of the blood-brain barrier in acute hepatic coma to a radiolabeled analog of GABA. [E. A. Jones, D. F. Schafer, J. G. Waggoner, A. K. Thakur (NHLBI) and M. Horowitz (NCI)].

II. Studies of Cellular Immune Function in Primary Biliary Cirrhosis

The role of abnormal immune mechanisms in the pathogenesis and perpetuation of the hepatobiliary lesion of primary biliary cirrhosis (PBC) is being studied. Recent studies have been designed to determine whether there is a defect in immune regulation in patients with PBC. T lymphocyte-mediated help and suppression of pokeweed mitogen-induced immunoglobulin (Ig) synthesis by cultured B lymphocytes have been quantitated in vitro using sensitive radioimmunoassays to measure IgG and IgM. Cultures containing T cells and autologous B cells from normal individuals synthesized progressively larger amounts of Ig with increasing T:B cell ratio up to a ratio of 1:1 (helper T cell effect). As the T:B cell ratio was increased above 1:1 cultures synthesized progressively smaller amounts of Ig (suppressor T cell effect). In contrast to the data in normal individuals, cultures containing T cells and autologous B cells from patients with PBC synthesized progressively larger amounts of Ig with increasing T:B cell ratio up to the highest ratio studied (10:1). In other words, the inhibition of Ig synthesis found at high T:B cell ratios in normal subjects is not found in patients with PBC. Using T cells which had been irradiated to abolish selectively suppressor T cell function, helper T cell function was found to be similar for patients

with PBC and controls. Thus, in PBC the magnitude of suppressor T cell-mediated inhibition of Ig synthesis by autologous B cells is markedly decreased, whereas the magnitude of helper T cell-mediated augmentation of Ig synthesis by autologous B cells is normal.

To characterize further this defect of immune regulation in PBC Ig synthesis by B cells co-cultured with allogeneic T cells at different T:B cell ratios was determined. Ig synthesis by B cells from patients with PBC in the presence of T cells from normal subjects was similar to that by B cells from normal subjects in the presence of allogeneic T cells from normal subjects. However, T cells from patients with PBC induced much more Ig synthesis by B cells from normal subjects than did allogeneic T cells from normal individuals. These findings suggest that the deficient suppression of Ig synthesis in PBC is due to a T cell defect. Patient control experiments indicated that the suppressor T cell defect in PBC is not secondary to chronic cholestasis or hepatocellular disease per se and that the defect exhibits specificity for PBC among liver diseases.

It has been shown that T cells can be induced to proliferate when cultured with either autologous or allogeneic irradiated B cells. These phenomena are known as the autologous and allogeneic mixed leucocyte reactions (MLR), respectively. The recent finding by others that patients with systemic lupus erythematosus (SLE) exhibit deficiencies of both suppressor T cell function and the autologous MLR prompted studies of the MLR in PBC. For patients with PBC, the mean autologous MLR was found to be significantly less than that for controls, whereas the mean allogeneic MLR was similar to that for controls. Thus, as in SLE, deficiencies of both suppressor T cell function and the autologous MLR are found in PBC. These deficiencies could be due to a common defect of cell interactions involved in the generation of suppressor effector cells. Such a defect could play an important role in the pathogenesis of autoimmune diseases such as PBC. [E. A. Jones, S. P. James, C. O. Elson (NCI) and W. Strober (NCI)].

III. Studies of Alpha-1-Antitrypsin Metabolism and of Protease Inhibitor Phenotypes

Alpha-1-antitrypsin (α_1AT) has been isolated from subjects with α_1AT deficiency and the protease inhibitor (Pi) phenotype ZZ (Z protein) and from normal subjects with the Pi phenotype MM (M protein). Simultaneous metabolic turnover studies of the M and Z proteins have been conducted in normal subjects (PiMM) and patients with α_1AT deficiency (PiZZ) using ^{125}I and ^{131}I to label each of the two proteins, respectively. Preliminary analysis of the turnover data from these studies indicates that the fractional catabolic rate of the Z protein in patients with α_1AT deficiency is similar to that of the M protein in normal subjects. Furthermore, the fractional catabolic rates of the M protein in patients with α_1AT deficiency and of the Z protein in normal individuals differ little from the fractional catabolic rate of the M protein in normal individuals. These findings indicate that the low serum concentration of α_1AT in α_1AT deficiency is not due to hypercatabolism of α_1AT but is attributable to decreased hepatic release of α_1AT. They also indicate that the differences in molecular structure between the Z and M proteins, which involve both the carbohydrate

and protein moieties, do not affect the rate of catabolism of α_1AT to any appreciable extent.

To study the possible relationship between intermediate deficiency states of α_1AT, i.e. non-MM phenotypes, and the development of pulmonary disease, Pi phenotypes were determined in 52 patients with Sjogren's syndrome. The proportion of these patients who had Pi phenotypes other than MM was not significantly greater than that for populations of normal subjects. Mean values for tests of pulmonary function, including estimates of both restrictive lung disease and airway obstruction, in patients with the MM phenotype were not significantly different from corresponding means for patients with non-MM phenotypes. These findings suggest that non-MM phenotypes in patients with Sjogren's syndrome are not associated with an increased susceptibility to develop chronic obstructive pulmonary disease. [E. A. Jones, J. Vergalla, J. Karsh, J. E. Gadek (NHLBI) and R. G. Crystal (NHLBI)].

IV. Studies of Chronic Hepatitis B Surface Antigen (HBsAg) Carriers

Patients with histologically confirmed chronic HBsAg positive hepatitis are being studied. A total of 37 patients have been evaluated and 21 followed for at least one year. 20-30% of patients undergo spontaneous resolution of their chronic hepatitis disease activity each year (as assessed by serial determinations of serum levels of aminotransferases). In each instance the spontenous resolution of elevated serum aminotransferase levels has been accompanied by serological changes of hepatitis B virus markers. Most striking has been the disappearance of the hepatitis B e antigen ("e" or HBeAg) and seroconversion to anti-HBe concurrent with the fall to normal of serum aminotransferase levels.

These studies on the natural history of chronic type B hepatitis were initiated as a prelude to the start of trials of anti-viral agents for this condition. One such trial is currently underway using the antiviral agent, adenine arabinoside monophosphate (Ara-AMP). Patients selected to be treated with this agent have been carefully monitored and pre-screened to establish that they are not on the verge of a spontaneous remission. This is assured by demonstrating that patients have high and stable levels of hepatitis B virus - as assessed by serial serum determinations of HBsAg titer, HBeAg, DNA-polymerase and hepatitis B virus DNA. Eight suitable patients have agreed to receive treatment with Ara-AMP and are being admitted for a ten-day course of the drug. The effects of the drug on serum levels of aminotransferases and hepatitis B virus markers are being carefully assessed. Patients who refuse anti-viral therapy are also being followed and constitute informal controls for the treatment group. Persons who spontaneously remit are also being followed. Further liver biopsies will eventually be obtained from these patients to document the degree of resolution of the underlying chronic hepatitis.

Immunological studies of patients with chronic type B hepatitis are also being conducted. Specific cell mediated immunity to various hepatitis B virus antigens (HBsAg, HBeAg, HBcAg) is being evaluated by means of antigen-induced lymphocyte proliferation assays and by assays for in vitro

production of specific antibody by cultured stimulated lymphocyte populations. [J. H. Hoofnagle, G. M. Dusheiko, S. P. James, D. F. Schafer, J. Gerin (Georgetown University), S. Straus (NIAID), R. Johnson (NIAID), R. H. Purcell (NIAID), E. A. Jones].

V. Controlled Trial of Chlorambucil Therapy in Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is a uniformly fatal disease characterized by slowly progressive intrahepatic cholestasis due to a chronic non-suppurative destructive cholangitis involving the septal and larger interlobular bile ducts. The disease is associated with multiple defects of immunologic function, hepatic copper overload and a high incidence of various autoimmune diseases. Trials of corticosteroids, azathioprine and d-penicillamine have not shown that any of these agents effectively inhibit the progression of the disease or increase survival. Because certain chronic inflammatory diseases appear to respond favorably to alkylating agents but not to azathioprine a controlled randomized trial of an alkylating agent for the treatment of PBC has been initiated. The alkylating agent chosen for the trial was chlorambucil rather than cyclophosphamide because of the less severe toxicity associated with the use of the former drug. So far 14 patients have been admitted to the trial. Each patient undergoes a comprehensive medical evaluation prior to randomization and again after being in the trial for one and two years. Each evaluation includes determinations of indices of cholestasis and hepatocellular function, serum levels of lipoproteins, autoantibodies, immune complexes and bile acids, and a needle biopsy of the liver. In those receiving the drug, total white cell counts are being maintained between 1000 and 3000/mm^3 in the presence of relative lymphocytopenia [E. A. Jones, D. F. Schafer, J. H. Hoofnagle, S. P. James and G. M. Dusheiko].

VI. Studies of Chronic Non-A, Non-B Hepatitis

Patients with well-defined chronic non-A, non-B hepatitis are being evaluated. The natural history of this condition is being studied in conjuction with the NIH Clinical Center Blood Bank (Dr. Harvey Alter). Patients with HBsAg negative post-transfusion hepatitis whose serum levels of aminotransferases remain elevated for more than six months are being admitted for a thorough assessment. Studies include HLA typing, and serological assays for non-A, non-B antigens and antibodies. Unless contraindicated a needle biopsy of the liver is obtained and is subjected to light microscopy for staging of the chronic liver disease, immunofluorscent staining for various antigens and electron microscopy for virus-like particles. Fifteen such patients have been evaluated and 12 have undergone percutaneous liver biopsy. [J. H. Hoofnagle, H. J. Alter (CC), H. Denes (NIAID), G. M. Dusheiko, D. F. Schafer, S. P. James, S. M. Feinstone (NIAID) and E. A. Jones).

VII. Studies of the Uptake of Lipoproteins by Hepatic Cells

Studies are being designed to determine which of the major classes of hepatic cells (parenchymal, endothelial and Kupffer) are responsible for the uptake of different lipoproteins by the liver and to define which apoproteins mediate the uptake of lipoproteins by the different cell types. To facilitate the generation of suspensions of hepatic cells for these

studies rat livers are perfused in situ with a solution of collagenase. The basic technique has been modified by including in the perfusate soybean trypsin inhibitor to reduce tryptic damage to cell surface receptors and deoxyribonuclease to inhibit DNA-mediated aggregation of cells. A method has been developed to separate gently endothelial and Kupffer cells from erythrocytes and parenchymal cells which involves Percoll gradient centrifugation. Endothelial cells are separated from Kupffer cells by centrifugal elutriation. The degree of separation achieved appears to be satisfactory as judged by selective stains for Kupffer cells (e.g. tartrate resistant acid phosphatase and endogenous peroxidase). The viability of cell preparations is being assessed by measuring cell membrane potentials. The first ligand to be used in these studies is radiolabeled human low density lipoprotein. [E. A. Jones, J. Summerfield, J. Vergalla, E. Schaefer (NHLBI), B. Brewer (NHLBI)].

ANNUAL REPORT SUMMARY
DIGESTIVE DISEASE BRANCH

Phoenix Clinical Research Section

The Section conducts clinical and laboratory research relating to diabetes, obesity and cholelithiasis primarily in the American Indian populations of the Southwest. Many of the investigations are done in close collaboration with the Southwestern Field Studies Section of the Epidemiology and Field Studies Branch.

The lipoprotein patterns in Indian and Caucasian diabetics and non-diabetics are being extensively investigated. Diabetes is associated with a decrease in HDL cholesterol levels and an increase in LDL cholesterol levels in the subgroups analyzed thus far. While American Indians with diabetes have higher total cholesterol, LDL cholesterol and VLDL triglyceride levels than those without diabetes, the level of each of these lipids was shown to fall significantly following the induction of a remission of diabetes by dietary means with accompanying weight loss. Such remissions were associated with an increase in the ratios of HDL to LDL cholesterol and accompanying falls in total cholesterol levels and in triglyceride concentrations. Such changes would appear to be beneficial given that total cholesterol and LDL cholesterol levels are risk factors for the development of macrovascular disease, and that HDL levels or the HDL/LDL cholesterol ratio is negatively correlated with the development of macrovascular atherosclerotic disease.

A method has been developed for the quantification of HDL subfractions isolated by flotation. The American Indians, and the Pima Indians in particular, have a low rate of coronary artery disease in spite of their obesity and high prevalence of diabetes. The concentration of HDL subfractions will be compared in Pima Indians and in Caucasians to determine if there are differences in HDL subfractions associated with either diabetes or documented EKG abnormalities.

Lower total and LDL cholesterol levels have been observed in Indians and these have been associated with decreased LDL synthetic rate. To investigate lipoprotein metabolism further in this group, VLDL metabolism was studied using ^{3}H glycerol as an endogenous precursor of VLDL TG synthesis. VLDL-TG synthesis was significantly reduced in the Pima Indians compared to Caucasians of similar degrees of obesity. In addition the fractional catabolic rate of VLDL in the Pimas was significantly higher than in the Caucasian controls. When the relation between VLDL-TG metabolism and plasma insulin was examined, plasma insulin levels in the Pima were not correlated with any parameter of VLDL-TG metabolism. Thus in this population with low plasma lipids and reduced arteriosclerotic heart disease VLDL pools appear to be insensitive to the influence of body weight and hyperinsulinemia. The further investigation of the lower synthetic rates of TG in the Pima Indians and its coordination with the overproduction of cholesterol observed in studies of bile composition should help elucidate reasons for the relative rarity of coronary artery disease among the Pima Indians, including those with diabetes, and may perhaps shed light on why Caucasian diabetics are so prone to develop atherosclerosis.

Free fatty acid levels have been studied in Pima Indians with severe and mild diabetes and in obese non-diabetic Pimas, and obese non-diabetic Caucasians. It was shown that free fatty acid levels in the Pima Indian diabetics are exquisitely sensitive to insulin at a time when the same subjects are resistant to the glucose lowering effects of insulin. These observations may explain why obesity can be maintained and increased at a time when subjects are becoming glucose intolerant, and indicates how the maturity onset diabetic may be resistant to the glucose lowering effects of insulin, yet may fail to develop any evidence of ketosis due to retention of sensitivity to the antilipolytic effects of extremely small amounts of circulating or exogenous insulin. In addition, VLDL-TG synthetic rates were found to correlate with plasma free fatty acid levels. Thus the observed decreases in VLDL production may also be associated with this tendency to maintain adipocyte TG deposition.

Investigation of the mechanisms underlying remission of diabetes which accompanies carbohydrate restriction and weight loss have been continued. Recent onset diabetics show a selected loss of glucose induced insulin secretion at a time when they retain considerable responsiveness to amino acid stimulation. These findings indicate that one component of the diabetic state in the maturity onset diabetic is a loss of the glucose sensing capacity of the pancreas at a time when the insulin secreting capacity and responsiveness to other stimuli is substantially intact.

Weight reduction and carbohydrate restriction have been shown to be associated with a substantial increase in insulin responsiveness to an oral glucose load at a time when fasting glucose levels are essentially normal, but post load levels remain elevated. Since continuation of weight loss then results in a continuing improvement in glucose tolerance which is accompanied by a fall in the post challenge hyperinsulinemia, and since obese subjects without diabetes, but who are glucose intolerant, have a similar post load hyperinsulinemia which also diminishes markedly with weight loss, it is postulated that an important step in the development of maturity onset diabetes is the development of increasing resistance to the glucose lowering effects of insulin.

Studies of insulin receptors in the Pima Indians have shown that while specific binding is reduced in both the obese non-diabetic and obese diabetic, weight reduction is accompanied by an increase in specific binding in both groups to a similar degree. It is postulated, therefore, that the obese diabetic shows increased insulin resistance which is not primarily the result of an insulin receptor deficiency. This suggests that the basis of insulin resistance in obesity and diabetes is perhaps an intracellular abnormality, although the reduced specific binding of insulin to cellular receptors probably contributes secondarily to the likelihood of decompensation of glucose metabolism.

An improved method has been developed to measure in vivo resistance to insulin mediated glucose disposal in order to investigate the mechanism of the hyperinsulinemia and diabetes in the Pima Indians. Existent techniques were found to incompletely block insulin secretion. To completely block endogenous insulin release, we, therefore developed an infusion mixture of

glucose insulin and somatostatin. Studies have shown that endogenous insulin secretion (determined by C-peptide measurement) and glucagon remained suppressed throughout the infusion. The method is simple, safe and independent of endogenous insulin secretion.

We have employed this method to quantify in vivo resistance to insulin mediated glucose disposal in a group of subjects with a wide range of obesity but normal glucose tolerance. With similar steady state levels of plasma insulin (SSPI) maintained in all subjects, the height of the steady state plasma glucose (SSPG) concentration was considered an index of total body sensitivity. A positive correlation between SSPG and the degree of obesity [Body Mass Index (BMI)] was demonstrated. The fasting plasma insulin concentration correlated with BMI and SSPG. Thus, by adjusting insulin according to body weight and maintaining a constant glucose infusion, this method can be used to study subjects over a wide range of obesity.

This method has been used to determine whether the insulin resistance of obese glucose-intolerant subjects can be accounted for by obesity alone. These studies have indicated that obesity is not the only factor responsible for the increased resistance in the obese glucose intolerant subjects. This increased resistance to insulin action was found in the presence of similar insulin binding to mononuclear cells. It appears, therefore, that in obese Southwestern American Indians with glucose-intolerance, abnormalities beyond the site of insulin binding to the receptor may explain the observed increase in in vivo insulin resistance.

We have also sought to determine whether the Pima Indians with normal glucose tolerance have increased in vivo insulin resistance compared to weight-matched Caucasian subjects. The findings to date using this method indicate that there is no difference in in vivo insulin resistance between these two groups. For this study we have also employed the euglycemic clamp technique of Andres et al. Under euglycemic conditions there was no difference in insulin mediated glucose disposal between the two groups.

Other studies are in progress to determine insulin sensitivity in genetic prediabetic Pima Indian subjects and to investigate changes in insulin resistance after control of diabetes and/or dietary manipulations.

The striking prevalence of obesity and diabetes among the Pima Indians has raised certain questions regarding the pathogenesis of non-insulin dependent diabetes which are amenable to investigative approaches employing diet and exercise. The observation that the modern lifestyle of the present-day Pima involves a considerable decrease in energy expenditure when compared with the primitive Pima has led to the proposal that decreased physical activity may play an important role in the pathogenesis of diabetes and obesity in this population. We are presently characterizing the beta cell response to oral stimuli and the peripheral sensitivity to the action of insulin in both obese non-diabetic and diabetic Pima Indians subjected to a training program during strict weight maintenance (to eliminate confounding effects of weight change). In two studies completed to date we have found that the beta cell response to oral stimuli was unchanged by training. However, there was a dramatic fall in the insulin response to i.v. glucose in an obese non-diabetic, suggesting

improved insulin sensitivity. There was only a slight change however in the i.v. glucose tolerance data in a normal-weight diabetic and no improvement in insulin secretory capacity. The extension of these studies to include obese diabetics will document the effects of physical training on peripheral sensitivity to insulin in diabetics and thus provide an adjunctive tool to diet therapy in obese, non-insulin dependent diabetics.

Investigations have been initiated to determine the cellular basis of insulin resistance in the Pima Indians. For many of these studies the cultured fibroblast has been selected for investigation. Suitable cell lines have been established from normal and diabetic Pima Indians and the parameters of cell growth between the normal and diabetic Pima cells have been found not to differ. However, a systematic difference between the growth characteristics of Pima cells and Caucasian cells has been identified, but it is postulated that this difference has an ethnic basis, rather than being related primarily to the determinants of diabetes. No differences in growth of Pima cells could be induced by culture in the presence of high levels of insulin.

The insulin receptors on fibroblasts have been characterized in relation to their number and affinity, and it has been demonstrated that insulin is able to regulate receptor number in both Pima and control fibroblasts. The affinity of the insulin receptor shows a significant negative correlation with the BMI of the fibroblast donor. A similar observation has been made for the variation in affinity of insulin binding in vitro to monocytes from normal Pima subjects over a range of obesity.

Studies on diploid fibroblast insulin receptors indicated that insulin mediated down regulation was not affected by plasma from normal subjects. In addition, pre-incubation of cells in normal plasma produced no alterations in subsequent insulin binding. This apparent lack of interference by components of normal plasma with the mechanism for down regulation and subsequent insulin binding provides a useful system for detection of possible antagonists of insulin action in plasma from insulin resistant subjects. Using this technique there appears to be a factor in plasma from a lipoatrophic diabetic with severe insulin resistance which inhibits down regulation in normal fibroblasts. The factor in this patient's plasma is not a protease against insulin or an antibody against the insulin receptor. This technique will be used to screen plasma samples from insulin resistant Pima subjects.

The fibroblast insulin receptor has been solubilized and partially purified, and the solubilized receptor preparation showed binding affinity, pH optimum and analog specificity similar to that for whole cells. No differences in binding kinetics were observed between receptors solubilized from Pima diabetic and non-diabetic cells. However this system has the potential for coordinated study of insulin receptor biochemistry and function.

Physiologic concentrations of insulin have been shown to stimulate total glucose incorporation in the cultured fibroblast. While the transport of hexose in these cells has been shown to involve a large diffusion component, insulin causes a small, but significant increase in V-max without influencing the K_m of transport. Insulin has been found to act at several steps of glucose metabolism in the fibroblast. Fractionation of the incorporated

glucose indicated that under the conditions employed approximately 30% is converted to glycogen, 30% to the ribose in nucleotides and 40% to lactate in the medium. Insulin was observed to stimulate incorporation into all of these fractions.

The action of insulin on glycogen synthase activity has been examined directly. Physiological concentrations of insulin stimulate the I form of the enzyme without changing total activity. The magnitude of the response to insulin was increased upon glucose starvation. Glucose and insulin effects on the enzyme were additive, indicating the mechanism of regulation of glycogen synthase in the fibroblast is similar to that observed in fat and muscle. This system has been used to examine the effect of decreased pH and the presence of β-hydroxybutyrate, as observed during ketoacidosis. Insulin action on glycogen synthase was impaired at low pH and was related to both decreased binding affinity and also the influence of post receptor components. If pH was maintained, the presence of β-hydroxybutyrate had no influence on insulin action. Thus in diabetic ketoacidosis the decreased pH rather than the presence of ketone bodies may be the primary mediator of interference in insulin action.

The stimulation of glucose metabolism by physiologic levels of insulin has been compared in Pima and Caucasian cells. Several parameters of insulin action were measured including hexose transport and glycogen synthase. No differences between the non-diabetic and diabetic Pima cells or between the Pima and Caucasian cells have been demonstrated. Thus the impaired insulin sensitivity observed in in vivo studies of individuals with abnormal glucose tolerance may not be due to inherent genetic differences in sensitivity to insulin action but rather to the complex physiological changes associated with the hyperglycemic state. Future studies will combine measurements of insulin binding, receptor regulation, hexose transport and other intracellular responses to insulin. It is hoped that these will lead to a further understanding of the mechanism of insulin resistance and its cellular basis.

The fibroblast systems developed for study of insulin action are also being employed for the examination of other factors which influence cell glucose metabolism. Alloxan has been shown to result in a dose dependent initial enhancement of glucose incorporation followed by an inhibition. This dual action of alloxan occurred beyong the level of phosphorylation. Studies of CO_2 production using 1 and 6 position labelled ^{14}C glucose suggested that alloxan initially stimulated the pentose shunt and subsequently inhibited both the pentose shunt and the glycolytic-TCA path. Presently peroxide and alloxan are being compared in order to elucidate the mechanism of action of both of these compounds. These studies will yield further understanding of cell controls of carbohydrate metabolism which will aid in the interpretation of alterations observed in the diabetic or insulin resistant states.

Preliminary data has been obtained on $Na^+ + K^+$ ATPase activity in red cells from normal Caucasians and normal or obese Pima Indians. Obese Pimas have Mg^{++} dependence for maximum activity which is significantly different from thin Pimas or Caucasians. These studies will be pursued and $Na^+ + K^+$ ATPase activity will be measured in diploid fibroblasts, since confirmation of these

differences, may provide a possible explanation for the development of obesity in the Pima Indians.

Glycosylated hemoglobin concentrations have been measured in a large number of Pima Indians with a wide spectrum of age and glucose tolerance in conjunction with the ongoing epidemiological studies of the Southwestern Field Studies Section. The frequency distribution of glycosylated hemoglobin levels in Pima Indians age 25 and over was shown to be bimodal. The relationship between glycosylated hemoglobin levels and the bimodal frequency distributions of the fasting and two hour post load plasma glucose levels in the same subjects shown a high degree of concordance between the elevation of glycosylated hemoglobin levels and both fasting and two-hour glucose levels. The characteristics of changing glycosylated hemoglobin levels with increasing age has been documented. The relationship between the specific complications of diabetes and glycosylated hemoglobin levels have been examined and, as expected, there was a high correlation between elevated glycosylated hemoglobin level and the presence of complications. No evidence has been found that glycosylated hemoglobin levels discriminate better than the fasting or two-hour glucose measurements for this purpose. Whether or not glycosylated hemoglobin levels predict the future development of complications more effectively than the glucose levels, however, remains to be determined. Nevertheless, glycosylated hemoglobin provides an index of glycemia which may be useful in epidemiological studies where the performance of a glucose tolerance test or the determination of glucose levels in the fasting state is impractical.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 53001-10 DDB

PERIOD COVERED
October 1, 1979 to Sept. 30, 1980.

TITLE OF PROJECT (80 characters or less)

Studies of Membrane Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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3.2	1.6	1.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The broad categories which are included in the project are: 1) Characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

PHS-6040

The divalent cation ionophore, A23187, accelerates the movement of divalent cations across artificial as well as biological membranes. In pancreatic acinar cells and in gastric parietal cells, A23187 causes functional changes that have been attributed to the ability of the ionophore to increase the influx of calcium into the cell. Direct measurements of calcium fluxes, however, have shown that the abilities of A23187 to cause functional changes in acinar cells as well as parietal cells are due to the ability of A23187 to increase the outward movement of calcium.

The actions of theophylline are usually attributed to its ability to inhibit cyclic nucleotide phosphodiesterase. In pancreatic acinar cells, theophylline not only inhibits phosphodiesterase but also has anti-muscarinic cholinergic actions and modifies the action of various pancreatic secretagogues on calcium transport.

Each of the pancreatic secretagogues that acts by causing mobilization of cellular calcium can cause desensitization of pancreatic enzyme secretion. In contrast, those secretagogues whose actions are mediated by cyclic AMP do not cause desensitization of enzyme secretion. Bombesin and structurally related peptides cause desensitization that reflects changes at the receptor level. Cholecystokinin and muscarinic cholinergic agents cause desensitization that reflects changes at the same post-receptor step - probably reflecting changes in the abilities of secretagogues to cause mobilization of cellular calcium.

Publications

McCarthy, D. M., Peikin, S. R., Lopatin, R. N., Crossley, R. and Harpel, S.: H_2-receptor antagonists in gastric hypersecretory states. In (no Eds.): Cimetidine Proceedings of International Symposium on Histamine H_2-receptor Antagonists. Amsterdam, Excerpta Medica Foundation, 1978, Vol. 443, pp. 153-164.

Jensen, R. T. and Gardner, J. D. Receptors for secretagogues on pancreatic acinar cells: Different mechanisms of spareness. In Bonfils, S., Fromageot, P. and Rosselin, G. (Eds.): Hormone Receptors in Digestion and Nutrition. North Holland, Elsevier/North-Holland Biomedical Press, 1979, pp. 167-176.

Gardner, J. D. and Jensen, R. T.: Receptors for secretagogues on pancreatic acinar cells. Am. J. Physiol. 238: G63-G66, 1980.

Gardner, J. D. and Jensen, R. T.: The physiologic and pharmacologic significance of potentiating interactions among secretagogues. In Miyoshi, A. (Ed.): Gut Peptides: Secretion, Function and Clinical Aspects. Tokyo, Kodansha LTD. Elsevier North/Holland Biomedical Press, 1979, pp. 154-156.

Olinger, E. J. and Gardner, J. D.: Action of VIP and secretin on adenylate cyclase activity in acinar cells from guinea pig pancreas. Gastroenterology 77: 704-713, 1979.

McCarthy, D. M., Katz, S. I., Gazze, L., Waldman, T. A., Nelson, D. L. and Strober, W.: Selective IgA deficiency associated with total villous atrophy of the small intestine and an ortan-specific anti-epithelial cell antibody. J. Immunol. 120: 932-938, 1978.

Gardner, J. D. and Rottman, A. J.: Action of cholera toxin on dispersed acini from guinea pig pancreas. Biochim. Biophys. Acta. 585: 250-265, 1979.

Lee, P. C., Jensen, R. T. and Gardner, J. D.: Bombesin-induced desensitization of enzyme secretion in dispersed acini from guinea pig pancreas. Am. J. Physiol. 238: G213-G218, 1980.

Gardner, J. D., Walker, M. D. and Rottman, A. J.: Effect of A23187 on amylase release from dispersed acini prepared from guinea pig pancreas. Am. J. Physiol. 238: G458-G466, 1980.

Gardner, J. D. and Jensen, R. T.: Regulation of pancreatic enzyme secretion in vitro. In Johnson, L., Christensen, J., Grossman, M., Jacobson, E. D. and Schultz, S. G. (Eds.): Physiology of the Digestive Tract, New York, Raven Press, (In press).

Korman, L. Y., Walker, M. D. and Gardner, J. D.: The action of theophylline on secretagogue-stimulated amylase release from dispersed pancreatic acini. Am. J. Physiol. (In press).

Abdelmoumene, S. and Gardner, J. D.: Cholecystokinin-induced desensitization in dispersed acini from guinea pig pancreas. Am. J. Physiol. (In press).

Gardner, J. D., Abdelmoumene, S., Lee, P. C., Collins, S. M. and Jensen, R. T.: Secretagogue-induced desensitization in pancreatic acinar cells. In Ribet, A. (Ed.): Biology of Exocrine Pancreatic Cell. Amsterdam, Elsevier/North Holland, 1980 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53002-08 DDB

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TITLE OF PROJECT (80 characters or less)

Gastrointestinal Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Digestive Diseases Branch

SECTION

Section on Gastroenterology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro systems are being used to study the mechanism of action of gastrin, secretin, cholecystokinin, bombesin, substance P and vasoactive intestinal peptide with their specific membrane receptors.

Clinical investigations are directed toward developing alternative forms of therapy for and elucidating the pathogenesis of disorders characterized by ectopic production of gastrointestinal hormones (e.g., Zollinger-Ellison syndrome and pancreatic cholera).

PHS-6040

In cholecystokinin, the sulfated tyrosine in the seventh position from the C-terminus is important for biologic activity. Studies using various analogues of cholecystokinin indicate that the role of the tyrosine moeity is to position the sulfate ester at the proper distance from the backbone of the peptide chain. The chemical structure of the amino acid per se appears to be of relatively minor importance.

Studies using ^{125}I-labeled cholecystokinin have shown that pancreatic acinar cells possess approximately 9000 cholecystokinin receptors and that stimulation of enzyme secretion is maximal when approximately 50% of these receptors are occupied by cholecystokinin or a structurally related agonist. Surprisingly, butyryl derivatives of cyclic GMP or cyclic AMP antagonize the action of cholecystokinin by inhibiting competitively the interaction of the peptide with its membrane receptors.

Studies using ^{125}I-labeled bombesin have shown that pancreatic acinar cells possess approximately 5000 bombesin receptors and that maximal stimulation of enzyme secretion occurs when approximately 40% of these receptors are occupied by bombesin or a structurally related agonist. Approximately 30 different fragments and analogues of bombesin have been tested for biologic activity on pancreatic acinar cells and for each peptide, its potency reflects its affinity for the bombesin receptor.

With pancreatic acinar cells ^{125}I-labeled physalaemin can be used to detect a class of receptors that interact with physalaemin, substance P, eledoisin and kassinin. Maximal stimulation of pancreatic enzyme secretion by any one of these peptides requires occupation of all of the receptors. Physalaemin, eledoisin and kassinin are derived from amphibians and appear to constitute phylogenetic precursors of substance P.

In patients with Zollinger-Ellison syndrome, the histamine H_2-blocking agent, cimetidine, will abolish gastric acid secretion and, as a result, is a therapeutic alternative to total gastrectomy. With the development of the ability to control acid secretion in the Zollinger-Ellison syndrome, the major therapeutic focus has shifted to attempts to treat the underlying neoplastic disease. Although only a small number of patients have been treated to date, the combination of adriamycin and 5-fluorouracil has had beneficial effects on the tumor in patients with the Zollinger-Ellison syndrome.

Publications:

Gardner, J. D.: Biochemical basis of action of gastrointestinal hormones. World Journal of Surgery. 3: 407-413, 1979.

Gardner, J. D. and Jensen, R. T.: Actions of pancreatic secretagogues in vitro: unresolved issues. In Miyoshi, A. (Ed.): Gut Peptides: Secretion, Function and Clinical Aspects. Tokyo, Kodansha, LTD. Elsevier/North-Holland Biomedical Press, 1979, pp. 123-128.

Jensen, R. T. and Gardner, J. D.: Interaction of physalaemin, substance P and eledoisin with specific membrane receptors on pancreatic acinar cells. Proc. Natl. Acad. Sci. USA 76: 5679-5683, 1979.

Gardner, J. D. and Jensen, R. T.: Initial steps in the mechanisms of action of gastrointestinal peptides. In Beers, R. F., and Bassett, E. G. (Eds.): Polypeptide Hormones, 12th Miles International Symposium. New York, Raven Press, 1980, pp. 395-412.

Jensen, R. T. and Gardner, J. D.: Gastrointestinal peptides: receptors and mechanisms of action in pancreatic acinar cells. In Gross, E., and Meienhofer, J. (Eds.): Peptides: Structure and Biologic Function, Proceedings of 6th American Peptide Symposium. Rockford, Ill., Pierce Chemical Co., 1979, pp. 1001-1009.

Fang, M., Ginsberg, A. L., Glassman, L., McCarthy, D. M., Cohen, P., Geelhoed, G. W. and Dobbins, W. D.: Zollinger-Ellison Syndrome with diarrhea as the predominant clinical feature. Gastroenterology 76: 378-387, 1979.

McCarthy, D. M., Peikin, S. R., Lopatin, R. N., Spiegel, A., Marx, S. and Brennan, M.: Hyperparathyroidism - A reversible cause of cimetidine resistant gastric hypersecretion. Brit. Med. J. 1: 1765-1766, 1979.

Krejs, G. J., Orci, L., Conlon, M., Ravazolla, M., Davis, G., Raskin, P., Collins, S., McCarthy, D. M., Baetens, D., Rubenstein, A. and Unger, R. H.: Somatostatinoma Syndrome. New Eng. J. Med. 301: 285-292, 1979.

McCarthy, D. M., Peikin, S. R., Lopatin, R. N., Crossley, R. J. and Harpel, H.: H_2-receptor-antagonisten bei hypersekretion. Excerpta Medica ICS 443: 153-164, 1978.

Binder, H. J., Grossman, M. I. and McCarthy, D. M.: Cimetidine - A status report. Current Prescribing 5: 23-29, 1979.

McCarthy, D. M.: Peptic Ulcer - Antacids or cimetidine? Hospital Practice 14: 52-64, 1979.

McCarthy, D. M.: Humorally mediated diarrheas. Practical Gastroenterology 4: 33-40 and 79-80, 1980.

McCarthy, D. M.: Peptic Ulcer. A Booklet in the "Medicine for Layman" Series. Department of Health and Human Resources, National Institutes of Health Publication no. 80-1943. Washington, D.C., United Stated Government Printing Office, 1980, pp. 26.

McCarthy, D. M.: The place of surgery in the Zollinger-Ellison syndrome. New Engl. J. Med. 302: 1344-1347, 1980.

Bodanszky, M., Martinez, J., Walker, M., Gardner, J. D. and Mutt, V.: Cholecystokinin (pancreozymin) 5. Hormonally active desamino derivative of Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2. J. Med. Chem. 23: 82-85, 1980.

Binder, H. J., Lemp, G. F. and Gardner, J. D.: Receptors for vasoactive intestinal peptide and secretin on small intestinal epithelial cells. Am. J. Physiol. 238: G190-G196, 1980.

Jensen, R. T., Lemp, G. F., and Gardner, J. D.: Interaction of cholecystokinin with specific membrane receptors on pancreatic acinar cells. Proc. Natl. Acad. Sci. USA 77: 2079-2083, 1980.

Pandol, S. J., Korman, L. Y., McCarthy, D. M. and Gardner, J. D.: The beneficial effect of oral lithium carbonate in the treatment of pancreatic cholera syndrome. New Engl. J. Med. 302: 1403-1405, 1980.

Loveridge, N., Hoile, R. W., Johnson, A. G., Gardner, J. D. and Chayen, J.: The cytochemical section-bioassay of gastrin-like activity. J. Immunoassay (In press).

Gardner, J. D., Rottman, A. J., Fink, M. L., Natarajan, S. and Bodanszky, M.: Importance of the amino acid in position 15 of VIP and secretin in influencing their interactions with membrane receptors on pancreatic acinar cells. Endo. Japonica (In press).

Gardner, J. D., Walker, M. D., Martinez, J., Priestley, G. P., Natarajan, S. and Bodanszky, M.: The importance of the amino acid in position 27 of cholecystokinin in determining its biological activity on pancreatic acinar cells. Biochim. Biophys. Acta. (In press).

Jensen, R. T.: Verner-Morrison Syndrome. Gastroenterology: A Weekly Update (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 53004-08 DDB

PERIOD COVERED

October 1, 1979 to Sept. 30, 1980

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotide Mediated Functions

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COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro systems are being used to characterize the mechanism by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides.

Whether cyclic GMP is a mediator of the actions of hormones, neurotransmitters and paracrine mediators on their target tissues remains to be determined. In the pancreas 5 different classes of secretagogues cause mobilization of intracellular calcium, and this mobilization activates guanylate cyclase and increases cyclic GMP. Initially, it seemed likely that cyclic GMP mediated the actions of these secretagogues on pancreatic enzyme secretion, particularly because exogenous 8Br cyclic GMP also increased enzyme secretion. Recent studies, however, have indicated that cyclic GMP does not mediate the actions of secretagogues on pancreatic enzyme secretion. For example, although 8Br cyclic GMP increases pancreatic enzyme secretion, it does so by mimicing the action of endogenous cyclic AMP. In addition, agents such as nitroprusside or hydroxylamine, which cause direct activation of guanylate cyclase, cause a 50-fold increase in cellular cyclic GMP, but do not increase pancreatic enzyme secretion.

In dispersed gastric parietal cells the action of histamine on acid secretion is produced by its interaction with histamine H_2-receptors, activatio of adenylate cyclase and increased cellular cyclic AMP. There is a good correlation between the abilities of various agents to antagonize the binding of histamine and their abilities to inhibit the actions of histamine on adenylate cyclase, cyclic AMP and gastric acid secretion.

All of the agents that cause secretory diarrhea in man and animals are thought to do so by increasing cellular cyclic AMP. Recently it has become clear that calmodulin is in some way involved in the mechanism by which cyclic AMP causes net intestinal secretion. Based on this information we have used phenothiazines, which inhibit calmodulin, to treat patients with secretory diarrhea caused by neoplasms that secrete vasoactive intestinal peptide. We have also found that lithium carbonate can inhibit the action of various agents on adenylate cyclase in intestinal mucosa, and that lithium can be used to treat patients with humorally mediated secretory diarrhea.

Publications

Gardner, J. D.: Hormones and diarrhea. In Janowitz, H. D. and Sachar, D. B. (Ed.): Proceedings of the First International Colloquium in Gastroenterology, Frontiers of Knowledge in the Diarrheal Diseases. Upper Montclair, New Jersey, Projects in Health, Inc., 1979, pp. 406-416.

McCarthy, D. M. and Coleman, M. C.: Response of intestinal mucosa to gluten challenge in autistic subjects. Lancet ii: 877-878, 1979.

Gardner, J. D. and Rottman, A. J.: Evidence against cyclic GMP as a mediator of the actions of secretagogues on amylase release from guinea pig pancreas. Biochim. Biophys. Acta. 627: 230-243, 1980.

Batzri, S. and Gardner, J. D.: Action of histamine on cyclic AMP in guinea pig gastric cells: inhibition by H_1- and H_2-receptor antagonists. J. Mol. Pharmacol. 16: 406-416, 1979.

McCarthy, D. M., Nicholson, J. A. and Kim, Y. S.: Intestinal adaptation to changing composition of the normal diet. Am. J. Physiol. (In press).

Hyman, P. E., Breenan, M. F., Head, G. and McCarthy, D. M.: Hyperamylasemia, duodenal duplication and pleural effusions in hereditary spherocytosis. Digestive Diseases and Sciences, (In press).

Gardner, J. D.: Pathogenesis of secretory diarrhea. In Clinical Physiology Series. Disturbances in Intestinal Salt and Water Transport. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 53,500-07 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies of the Transport of Organic Anions by the Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: E. A. Jones Acting Chief, Liver Diseases Section DDB:NIAMDD

Other: J. G. Waggoner Chemist DDB:NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/4	1/4	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The hepatic uptake and net hepatic clearance of conjugated bilirubin in rats is substantially faster than that of unconjugated bilirubin. An appreciable affinity of conjugated bilirubin for intrahepatic binding proteins, such as ligandin, has been demonstrated. The existence of a significant intrahepatic storage pool for bilirubin monoglucuronide, but not bilirubin diglucuronide, has also been established. These observations may imply that the formation and excretion of bilirubin diglucuronide occurs directly from the canalicular pool of the hepatocyte. Saturable binding of sulfobromophthalein to hepatic canalicular and plasma membranes has been demonstrated. Furthermore, proteins with high affinities for bilirubin and BSP have been isolated from hepatic cell surface membrane fractions suggesting that carrier-mediated transport mechanisms may be of importance in the hepatic uptake and excretion of these organic anions. In studies of bile salt transport it has been shown that microfilaments play a role in hepatic active transport systems and that the excretion of currently unidentified anions contribute to the bile salt independent fraction of bile in the rat.

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Project Description:

Objectives:

1) To study the transport of organic anions from blood across the hepatocyte into bile, in order to characterize the biophysical nature of each of the transport steps involved in the overall hepatic excretory system.

2) To develop a capability for quantitating the function of each of these steps in patients with hepatic dysfunction by analysis of kinetic studies of various test substances such as sulfobromophthalein (BSP), indocyanine green, radiolabeled bilirubin and radiolabeled bile salts.

Methods Employed:

1) Plasma disappearance curves of labeled or unlabeled organic anions are analyzed using appropriate computer programs.

2) Pure preparations of biosynthetically labeled bilirubin conjugates are isolated from rat bile using albumin-agarose affinity columns.

3) Homogenates and cytosol of liver are subjected to thin layer chromatographic analysis of ^{3}H-labeled bile pigments after administrating unconjugated ^{3}H-bilirubin intravenously to rats. The dissociation constants of bilirubin and its conjugates for glutathione-S-transferase are measured fluorometrically.

4) Rat liver plasma and canalicular membrane fractions are prepared by differential and isopycnic centrifugation.

5) Proteins with affinities for BSP and bilirubin are isolated from rat liver membrane fractions by affinity chromatography.

6) A rapid filtration technique is used to separate cells from medium in studies of the initial uptake velocity of taurocholate by hepatocytes.

7) Polyacrylamide gel electrophoresis is used in the assessment of the effect of bile salts on protein excretion into bile.

Major Findings:

1) Fasting and postpranial serum bile acid concentrations and plasma disappearance rates of injected cholyl-glycine are entirely normal in all three subtypes of Gilbert's syndrome. These findings confirm previously fragmentary information which suggests that the transhepatic transport of bile acids occurs over separate pathways from those involved in the excretion of other cholephilic anions. They are also of clinical significance in that even minimal structural hepatic disease has been found almost invariably to be associated with an abnormality in either the postprandial serum total concentration of bile acids or plasma bile acid disappearance rates. Hence,

in patients with subtypes of Gilbert's syndrome associated with abnormalities of BSP metabolism normal bile acid studies strongly mitigates against the existence of structural liver disease and can be used as a means of obviating the necessity for invasive procedures such as liver biopsy in these patients.

2) An analysis of plasma disappearance curves of injected cholyl-glycine in normal subjects as assessed by a radioimmunoassay, which was reported by others, resulted in values for the initial volume of distribution that exceeded that of total body water and values for clearance from the plasma which exceeded hepatic blood flow. These findings prompted a further study of the disappearance of injected cholyl-glycine from plasma in normal subjects in which measurements of cholyl-glycine were made using the 3-hydroxysteroid dehydrogenase method. This study indicated that the rate of disappearance of unlabeled cholyl-glycine from plasma was the same as that of ^{14}C-labeled cholyl-glycine. This rate was similar to the corresponding rate for the more extensively studied cholephilic anion indocyanine green. Calculated values for the initial volumes of distribution of labeled or unlabeled cholylglycine appoximated to the plasma volume.

3) To obtain new insights into the relationship between plasma bilirubin turnover and hepatic bilirubin clearance, studies of bilirubin kinetics have been conducted in patients with hereditary spherocytosis before and after splenectomy. In patients whose pre-operative bilirubin clearance values were normal, the fall in bilirubin production occurring after splenectomy had no effect on hepatic bilirubin clearance, whereas in patients whose pre-operative bilirubin clearance values were low and in the range found in Gilbert's syndrome, there was a marked increase in hepatic bilirubin clearance following surgery. These findings suggest that in the patients with Gilbert's syndrome the hepatic bilirubin transport system was operating at a level close to saturation and that bilirubin loading studies may prove to be a useful provocative test for the diagnosis of carriers of the Gilbert's syndrome gene who do not express the trait at basal levels of bilirubin production.

4) The previous isolation of a pure preparation of biosynthetically labeled bilirubin conjugates in this laboratory has enabled kinetic studies of the hepatic metabolism of conjugated bilirubin to be carried out in rats. It has been shown that values for both hepatic uptake and net hepatic clearance of conjugated bilirubin are substantially faster than corresponding values for unconjugated bilirubin, presumably due, at least in part, to the ability of the former pigment to bypass the conjugation step. In addition, the hepatic uptake of conjugated bilirubin, like that of the unconjugated pigment, was inhibited by other organic anions, such as BSP and indocyanine green, but not by glycocholate, suggesting that the hepatic uptake of conjugated bilirubin is not mediated by simple diffusion but by a more complex mechanism shared with unconjugated bilirubin.

5) Bilirubin monoglucuronide accumulates preferentially within the hepatocyte whereas the diglucuronide appears to be preferentially secreted

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into bile. In addition, unconjugated and conjugated bilirubins were found to have a high affinity for glutathione-S-transferases such as ligandin. Based on these findings it is suggested that microsomal monoconjugation of bilirubin may occur while it is bound to a glutathione-S-transferase and that the biliary excretion of bilirubin may involve interaction of a bilirubin monoglucuronide glutathione-S-transferase complex with the bile canalicular membrane with resultant excretion of bilirubin diglucuronide directly from the canalicular pool of the hepatocyte.

6) To assess the potential role of membrane carriers in the hepatic transport of organic anions, the kinetics of binding of labeled BSP to canalicular and plasma membrane fractions isolated from rat hepatocytes were studied. Saturable binding of BSP to both membranes was shown and this binding was competitively inhibited by bilirubin and indocyanine green but not by taurocholate. This investigation has provided further support for the hypothesis that organic anions other than bile salts share a common pathway for hepatic uptake and biliary excretion and that membrane carrier proteins may be involved in these processes.

7) Potential carrier proteins have been isolated from sinusoidal and canalicular enriched liver membrane fractions using affinity chromatography. From BSP affinity columns three binding proteins with apparent molecular weights of 4.0, 4.6 and 6.0 x 10^4 were eluted. From bilirubin affinity columns a single binding protein with an apparent molecular weight of 6.0 x 10^4 was eluted. Thus, one or more membrane carrier proteins may be involved in the hepatic transport of bilirubin and organic anionic dyes.

8) To investigate whether elevated fasting and post-prandial serum bile salt concentrations in patients with chronic liver disease might be due, at least in part, to abnormal bile salt transport, uptake of taurocholate by hepatocytes isolated from normal rats and rats with fibrosis induced by carbon tetrachloride was determined. Taurocholate uptake by hepatocytes from rats with hepatic fibrosis was noncompetitively inhibited, raising the possibility of decreased numbers of receptors for bile salts on these cells.

9) In a study of the differential effects of taurolithocholate and taurocholate on protein excretion into bile in rats, it has been shown that taurocholate induces some loss of surface proteins of the canalicular membrane without affecting the integrity of this membrane. In contrast, taurolithocholate induced substantially more protein excretion and this was associated with loss of integral canalicular membrane and Golgi components into bile. These findings may be related to the observed loss of canalicular microvilli and cholestasis caused by taurolithocholate.

10) The role of microfilaments and microtubules in bile salt transport has been studied in rats by assessing the effects of cytochalasin B, a microfilament inhibitor, and colchicine, a microtubule inhibitor, on taurocholate uptake by isolated rat hepatocytes. Cytochalasin B competitively inhibited taurocholate uptake, implying either binding of cytochalasin B to a receptor for taurocholate or direct participation of microfilaments in taurocholate uptake. Colchicine noncompetitively inhibited taurocholate uptake

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suggesting either an indirect or no role of microtubules in taurocholate transport. Thus, microfilaments may be of importance in active transport systems of the hepatocyte and their functional impairment may lead to cholestasis by inhibiting bile salt transport.

11) In a further study of bile salt transport, an explanation of the anion gap in rat bile was sought. Bile salt-depleted bile was found to increase hepatocellular bile flow in a dose-dependent fashion without altering the permeability of the biliary tree. The unidentified choleretic agent was heat stable and was not a bile salt. This finding suggests that part of the bile salt independent fraction of bile may be dependent on the excretion of currently undefined anions.

Significance to Biomedical Research:

Studies of organic anion kinetics in patients with Gilbert's syndrome, Crigler-Najjar syndrome and normal volunteers have led to a marked improvement in our understanding of the excretory function of the liver. In particular, these studies have demonstrated that it is possible to quantitate hepatic function in terms of clearance values in a manner analagous to the use of creatinine clearance measurements for quantitating renal function. The ability to accurately measure both bilirubin turnover and bilirubin clearance, the two parameters which determine the plasma bilirubin concentration, has made possible a precise functional classification of all cases of unconjugated hyperbilirubinemia. Studies of the sort described above are also leading both to the development of new clinical methods for assessing the function of individual steps in organic anion transport processes and to an improved understanding of the nature of the defects in a variety of disease states which affect the liver.

Proposed Course:

This project is currently inactive. Future studies might include the following:

1) The standard protocol for defining the disappearance from plasma of intravenously administered radiolabeled unconjugated bilirubin could be extended by counting radioactivity in the upper layers of the Weber-Schalm partition of plasma samples. To the extent that the Weber-Schalm system effectively separates unconjugated from conjugated bilirubin, such data would reflect incorporation of the radiolabel into plasma conjugated bilirubin. Data of this type could be subjected to conventional curve fitting and mathematical modeling techniques.

2) Preparations of pure radiolabeled conjugated bilirubin could be used to study the kinetics of the conjugated pigment in vivo in experiments having similar designs to analogous studies that have already been conducted using radiolabeled unconjugated bilirubin.

3) A distributed modeling appraoch should be incorporated into the experimental design and analysis of future studies of bilirubin metabolism in vivo if insights into blood transport delays, short term effects within the

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hepatic circulation and gradients of concentration of unconjugated and conjugated bilirubin from the portal to the centrilobular zone of the hepatic lobule are to be obtained.

Publications:

1. Reichen, J., Berk, P.D.: Isolation of an organic anion binding protein from rat liver plasma membrane fractions by affinity chromatography. Biochem. Biophys. Res. Commun. 91: 484-489, 1979.

2. Berk, P.D., Jones, E.A., Howe, R.B., Berlin, N.I.: Disorders of bilirubin metabolism. In Bondy, P.K. and Rosenberg, L. (Eds): Metabolic Control and Disease, ed. 8. Philadelphia, W. B. Saunders and Co., 1979, pp. 1009-1088.

3. Jones, E.A., Berk, P.D.: Liver function. In Brown, S.S., Mitchell, F.L. and Young, D.S. (Eds.): Chemical Diagnosis of Disease. Amsterdam, Elsevier/North Holland, 1979, pp. 525-662.

4. Summerfield, J.A., Scott, J., Berman, M., Ghent, C., Bloomer, J.R., Berk, P.D., Sherlock, S.: Benign recurrent intrahepatic cholestasis: studies of bilirubin kinetics, bile acids and cholangiography. Gut 154: 154-160, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,501-07 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies of the Pathogenesis of Acute Hepatic Coma and the Use of Artificial Hepatic Support Devices for its Treatment

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
Other:	D. F. Schafer	Clinical Associate	DDB:NIAMDD
	M. L. Zeneroli-Baraldi	Visiting Scientist	DDB:NIAMDD
	J. G. Waggoner	Chemist	DDB:NIAMDD
	A. K. Thakur	Investigator	RR:NICHD
	P. J. Munson	Investigator	RR:NICHD
	M. Horowitz	Clinical Associate	POB:NCI

COOPERATING UNITS (if any)
RR:NICHD
POB:NCI

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1 1/2	1/2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An animal model of acute hpeatic failure which closely resembles human fulminant hepatic failure has been developed in an inbred strain of rabbits using the selective hepatotoxin galactosamine. An extracorporeal hepatic support system for the removal of potentially encephalopathic substances from the plasma of rabbits has been developed. If certain specific surfaces are incorporated into this device, hemoperfusion results in complement activation, the pulmonary sequestration of aggregated neutrophils and death. Large doses of hydrocortisone do not prevent this syndrome. The recording of repeated visual evoked potentials in the central nervous system is a reliable, quick, non-invasive method of quantifying neurologic changes in hepatic encephalopathy. Serum levels of γ-aminoburytic acid (GABA), a major inhibitory neurotransmitter, are increased in acute hepatic coma. The main source of GABA in peripheral blood is the enteric bacterial flora. The density of receptors for GABA on postsynaptic neural membranes is increased in acute hepatic coma.

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Project Description:

Objectives:

1) It is not established whether hepatic coma is due to the accumulation of an encephalopathic substance which is inadequately cleared by the diseased liver or to a failure of the diseased liver to synthesize an adequate amount of a substance necessary for normal brain function. A major objective of this study is to obtain new insights into the pathogenesis of hepatic coma. In particular, the object is to identify and characterize potential encephalotoxins in acute hepatic coma, and to determine whether they cross the blood-brain barrier. If they induce neural inhibition their site and mode of action will be investigated.

2) An additional objective is to develop extracorporeal hemoperfusion circuits for removing from plasma those metabolic products which are potentially encephalopathic and which are ordinarily extracted by the liver. In applying this approach the effects of including different adsorbents in the circuit and the effects of different hemoperfusion schedules will be assessed. The ultimate objective of this project is the development of a biocompatible artificial liver support system for use in the management of acute potentially-reversible hepatic failure.

Methods Employed:

1) To generate an animal model of fulminant hepatic failure, the selective hepatotoxin, galactosamine-HCl, is administered intravenously according to a precise dose schedule to an inbred strain of rabbits.

2) The clearance (ml/min) of potentially encephalopathic metabolites by columns containing a variety of adsorbents has been measured using plasma supplemented with labeled and unlabeled metabolites in a simple recirculation system.

3) Rabbits in hepatic failure have been treated by perfusing their blood over a biocompatible microporous polypropylene membrane and passing the dialysate over a column of an adsorbent before recirculating it over the membrane.

4) Visual evoked potentials in the central nervous system are measured via chronically implanted epidural electrodes. Methods have been standardized for electrode implantation and data recovery for normal rabbits and rabbits in hepatic coma and other types of encephalopathy. Analysis of repeated recordings is performed by a dedicated computer system.

5) The concentration of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) is measured in serum and other fluids using a standard radioreceptor assay. The basis of this assay is the ability of cold GABA to displace [^{3}H]-GABA from specific receptors on preparations of postsynaptic neural membranes isolated from brain. The densities and affinities of GABA receptors on neural membranes are determined by computer assisted analysis of Scatchard plots.

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6) The porosity of the blood-brain barrier to a radiolabeled analog of GABA is being studied in the rabbit model of fulminant hepatic failure using computer enhanced autoradiography.

Major Findings:

1) A galactosamine-induced animal model of fulminant hepatic failure has been generated in an inbred strain of rabbit which has the advantages of being non-hazardous to personnel and reproducible. The model closely resembles human fulminant hepatic failure in its clinical, electroencephalographic, serum biochemical and hepatic histologic features. Death occurs from liver failure. Cross circulation experiments between the model and a normal rabbit indicate that the model is potentially reversible. The rabbit is sufficiently large to permit studies of the effects of extracorporeal hemoperfusion devices on fulminant hepatic failure.

2) Arousal of patients from coma due to fulminant hepatic failure may be attributable, at least in part, to depletion of protein-bound substances toxic to the brain from a slowly exchanging extravascular pool such as the brain or CSF. The hypothetical effects of different hemoperfusion schedules on the kinetics of depletion of a protein-bound substance, unconjugated bilirubin, have been simulated using a computer. Data on the kinetics of unconjugated bilirubin in man and experimentally determined values for the efficiency of hemoperfusion through albumin-agarose columns in removing unconjugated bilirubin from plasma were used in the simulations. The results suggest that simple alterations in the schedule of hemoperfusions might accelerate depletion of toxins from an extravascular pool and hence promote earlier arousal and possibly more frequent survival in patients with fulminant hepatic failure.

3) Columns containing various activated carbons are ineffecient at clearing dicarboxylic amino acids and short chain fatty acids from plasma. In contrast, columns containing granular and spherical carbons are very effective at clearing from plasma aromatic amino acids and certain neurotransmitters, substances of potential importance in the pathogenesis of hepatic coma. Columns containing Dowex 20, Dowex 50, BioBead SM-2 and LH-20 are ineffective at removing a representative series of potentially encephalopathic metabolites.

4) Normal rabbits, subjected to hemoperfusion schedules in which blood is perfused through silicone rubber tubing or over columns of carbon embedded in hydroxyethylcellulose, develop hyperventilation and respiratory alkalosis and subsequently die. This hemoperfusion-induced respiratory distress syndrome is associated with polymorphonuclear leukopenia and the sequestration of aggregated polymorphonuclear leukocytes in pulmonary arterioles. A heat labile factor in plasma, which is probably the activated complement component C5a, is necessary for the development of this syndrome. Hemoperfusion through silastic tubing is not associated with this syndrome. Corticosteroids inhibit C5a-mediated aggregation of polymorphonuclear leukocytes in vitro. However, when the blood of rabbits was perfused over an implicated foreign surface, hydrocortisone 150 mg IV per kg per hour did not prevent the pulmonary sequestration

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of aggregated polymorphonuclear leukocytes and a fatal respiratory distress syndrome.

5) A hemoperfusion system has been devised which avoids problems of bioincompatibility due to interaction between formed elements of the blood and adsorbents, and embolization of particles of adsorbent. Blood is perfused over a microporous polypropylene membrane. The dialysate, which is in a closed circuit, is recirculated over the membrane after passage through a column of adsorbent. This arrangement avoids disadvantages associated with fluid shifts across the membrane. Normal rabbits subjected to 4-hour periods of hemoperfusion using this system have shown no ill effects.

6) The recording of visual evoked potentials has been shown to be a reliable quick, non-invasive method for quantifying neurologic changes in hepatic encephalopathy. The pattern and amplitude of visual evoked potentials associated with acute hepatic coma are similar to those associated with coma induced by certain hypnotic agents, e.g. phenobarbital and diazepam, but not others, e.g. ether. As barbiturates and benzodiazepines are known to interact with postsynaptic receptors for GABA in the brain, it is possible that putative encephalotoxins in hepatic encephalopathy exert their effects via the GABA innibitory neurotransmitter system.

7) Studies involving the application of the GABA radioreceptor assay have shown: (i) A major source of GABA in peripheral blood is the enteric bacterial flora; (ii) Levels of GABA in peripheral blood are increased in acute hepatic coma but not in another type of metabolic encephalopathy; and (iii) The density of receptors for GABA on postsynaptic neural membranes of the brain is increased in acute hepatic coma. These findings suggest that in hepatic coma GABA derived from the gut crosses a porous blood-brain barrier and induces its own receptors in the brain. This hypothesis affords potential explanations of the generalized neural inhibition of hepatic encephalopathy and of increased sensitivity of the brain to certain hypnotic drugs in hepatic encephalopathy.

Significance to Biomedical Research:

The mortality rate from fulminant hepatic failure of various etiologies in man is usually 80-90%. Various types of temporary hepatic support have been used in the treatment of this syndrome in uncontrolled studies in the hope of buying time to allow hepatic regeneration to take place. Such attempts at taking over the functions of a failing liver have, to date, had little if any effect on the mortality rate. Advances in this field are impeded by continuing uncertainties regarding the pathogenesis of coma in fulminant hepatic failure. It is not clear whether coma is due to toxic substances which impair cerebral function or to the lack of a hepatic-synthesized substance which is necessary for normal cerebral function. Furthermore, there are no reliable clinical or laboratory findings which enable the clinician to identify those patients in coma from fulminant hepatic failure who will die irrespective of any treatment modality, those who will recover spontaneously and those whose life might be saved by providing temporary hepatic support. Further insights into the pathogenesis of fulminant hepatic failure are being sought by studying the metabolism of inhibitory neurotransmitters and the status of receptors for

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inhibitory neurotransmitters in acute hepatic coma. In addition, the availability of an animal model of fulminant hepatic failure enables controlled studies of the efficacy of different types of temporary hepatic support device to be conducted. The results of such studies together with a better understanding of the pathogenesis of hepatic encephalopathy should lead to more effective management of human fulminant hepatic failure.

Proposed Course:

In the immediate future futher studies of the metabolism of GABA and the status of the GABA inhibitory neurotransmitter system in acute hepatic coma will be conducted. These studies will include: (i) An assessment of the timing and significance of increased porosity of the blood-brain barrier in relation to the onset of acute hepatic coma; (ii) Determination of the activity of the hepatic enzyme which metabolizes GABA, GABA transaminase, in the rabbit model of fulminant hepatic failure; (iii) Measurements of the binding of analogues of GABA to GABA receptors; (iv) Measurements of the serum level of GABA in patients with hepatic encephalopathy and other metabolic encephalopathies; and (v) Determination of the densities of receptors for GABA in the brains of patients who have died from hepatic coma and other causes.

Publications:

1. Zeneroli, M.L., Waggoner, J.G., Schafer, D.F., Kolobow, T., Jones, E.A.: Development of an extracorporeal hepatic support device: Clearance of amino acids short chain fatty acids and neurotransmitters by different carbons. In (no eds.) Aminoacidi ed Encefalopatia Epatica. Pisa, Pacini Editore, 1979, pp. 81-93.

2. Berk, P.D., Schafer, D.F., Blitzer, B.L., Jones, E.A.: Computer models and animal models: The rational stepwise approach to the development of artificial hepatic support systems for the treatment of fulminant hepatic failure. In Sideman, S. and Chang, T.M.S. (Eds.): Hemoperufusion: Kidney and Liver Support and Detoxification. Washington, D.C., Hemisphere Publishing Corp., 1980, pp. 151-174.

3. Zeneroli, M.L., Schafer, D.F., Waggoner, J.G., Jones, E.A.: Hemoerfusion-induced respiratory distress syndrome in an animal model: The effects of corticosteroids. In Brunner, G. (Ed.): Artificial Liver Support. Berlin, Springer-Verlag, 1980 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,503-06 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunologic Studies in Chronic Active Hepatitis and Primary Biliary Cirrhosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
Other:	S. P. James	Clinical Associate	DDB:NIAMDD
	W. Strober	Senior Investigator	MET:NCI
	C. O. Elson	Expert	MET:NCI

COOPERATING UNITS (if any)

Metabolism Branch, NCI

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 3/4	1 1/2	1/4

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Abnormal immune mechanisms are being studied in patients with HBsAg negative chronic active hepatitis and primary biliary cirrhosis (PBC) selected according to strict diagnostic criteria. The in vitro cytotoxic capacity of lymphocytes and lymphocyte subpopulations (T, K and B cells) against a hepatic target cell and a non-hepatic target cell in tissue culture has been evaluated. T cell mediated help and suppression of pokeweed mitogen-induced immunoglobulin synthesis by B cells has been studied using radioimmunoassays to measure IgG and IgM synthesized by cultures of appropriate mixtures of lymphocyte subpopulations in vitro. The ability of T cells to proliferate when cultured with either autologous or allogeneic irradiated B cells (mixed lymphocyte reactions) has been assessed. Results of these studies include the demonstration in PBC of (i) reduced spontaneous cytotoxic function of a subpopulation of lymphocytes which lack surface immunoglobulin but bear Fc receptors; (ii) diminished capacity of T cells to inhibit immunoglobulin synthesis in vitro; and (iii) a deficiency of the autologous but not the allogeneic mixed lymphocyte reaction.

PHS-6040
(Rev. 10-76)

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Project Description:

Objectives:

Patients with HBsAg negative lupoid chronic active hepaittis and primary biliary cirrhosis (PBC) exhibit striking defects of immunologic function. Currently immunologic mechanisms are considered to play major roles in the pathogenesis of these two diseases. The main objective of this study is to evaluate in detail the immunologic status of patients with these two diseases. In particular, immunologic phenomena which are likely to be of pathogenic significance are studied. The ultimate goal is to identify the major pathogenic mechanisms in both diseases.

Methods Employed:

Criteria for patient selection are based on history, physical examination, results of serum biochemical tests, serum non-organ specific antibody titers and hepatic histology. Patients with a history of ingestion of hepatotoxic drugs are excluded. To date the study has been limited to HBsAg negative subjects. A few patients with alcoholic liver disease have been studied to generate control data in a chronic liver disease in which the etiology is not primarily immunologic. Similarly, a few patients with large duct biliary obstruction and sclerosing cholangitis have been studied to generate control data in patients who have cholestasis not due to PBC.

1) The immunologic status of patients is assessed by multiple intradermal antigen tests, primary immunization with keyhole limpet hemocyanin and pneumococcal polysaccharide with subsequent keyhole limpet hemocyanin skin testing, quantitation of T and B cell populations using rosetting and immunofluorescence techniques, quantitation of serum concentrations of immunoglobulins and complement components and functional (hemolytic) assays of complement components. Patients are also HLA typed.

2) For immunologic studies using peripheral blood mononuclear cells (PBMC), PBMC are harvested from blood by Ficoll-Hypaque density gradient centrifugation. T cell and non-T cell fractions are separated using Sephadex anti-$(Fab')_2$ immunoabsorbant affinity columns. PBMC are depleted of monocytes by removing cells which adhere to plastic, by removing cells which ingest iron particles by application of a magnetic field or by passing cells over Sephadex G-10 columns. Cell fractions are characterized by determining the proportions which form rosettes with sheep erythrocytes, have surface immunoglobulin detected by immunofluorescence, ingest latex beads, and are adsorbed onto a surface coated with insoluble immune complexes.

3) The cytotoxic capacity of lymphocytes and lymphocyte subpopulations harvested from peripheral blood is assessed in an in vitro cytotoxicity assay, using target cells which include Chang human hepatoma cells and mouse EL-4 sarcoma cells. The assay involves quantitating ^{51}Cr released from labeled target cells by lymphocytes from normal subjects and patients run in

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parallel. The technique is used to assay spontaneous cell-mediated cytotoxicity (SCMC), mitogen induced cellular cytotoxicity (MICC) (using phytohemagglutinin, concanavalin A and pokeweed mitogen) and antibody dependent cellular cytotoxicity (ADCC) (using an appropriate anti-target cell antibody).

4) The capacity of the reticuloendothelial system to remove from the circulation particles by non-specific phagocytosis and immunospecific mechanisms is assessed. Nonspecific phagocytosis is determined from the rate of disappearance of a test dose of ^{125}I-microaggregated albumin from plasma and hepatic blood flow from the rate of disappearance of a tracer dose of this substance from plasma. Immunospecific clearance is investigated by following the fate of intravenously administered autologous red cells labeled with ^{51}Cr and coated with a pure IgM or IgG antibody directed against ABO or Rh antigens, respectively. Clearance of IgM coated cells occurs largely in the liver and depends on the interaction between C3b deposited in vivo on the red cell membrane and specific C3b receptors on Kupffer cells, while clearance of IgG coated cells is mediated by Fc-receptor-bearing fixed macrophages in the spleen. After clearance IgG-coated cells undergo phagocytosis, whereas IgM-coated cells either undergo phagocytosis or release back into the circulation as C3d-coated cells as a result of the action of circulating C3b inactivator.

5) The regulation of immunoglobulin synthesis by B cells is studied by quantitating T cell mediated help and suppression of pokeweed mitogen-induced immunoglobulin synthesis in vitro. This is achieved by culturing B cells with unirradiated or irradiated T cells at different T:B cell ratios and measuring IgG and IgM synthesis by the cultured B cells using sensitive radioimmunoassays. Immunoglobulin synthesis by autologous and allogeneic mixtures of T and B cells from patients and control subjects are studied.

6) The ability of T cells to proliferate when cultured with either autologous or allogeneic irradiated non-T cells is determined. These phenonema are known as the autologous and allogeneic mixed lymphocyte reactions (MLR), respectively. Incorporation of ^{3}H-thymidine into T cells is used as an index of cell proliferation.

Major Findings:

1) There is a higher incidence of skin test anergy in patients with PBC than in patients with chronic active hepatitis and controls. This anergy includes the response to de novo immunization with keyhole limpet hemocyanin.

2) The cytotoxic response in patients with PBC and HBsAg negative chronic hepatitis is not specific for the Chang cell and does not indicate prior sensitization in vivo to antigens on this target cell.

3) In contrast to chronic hepatitis, SCMC of lymphocytes is defective in PBC. There is no defect in MICC or ADCC in either disease. In both diseases SCMC and ADCC are mediated by a subpopulation of lymphocytes which lack surface immunoglobulin and bear Fc receptors, whereas MICC is mediated

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by lymphocytes which lack both surface immunoglobulin and Fc receptors. Thus, there is a difference in cytotoxic function between PBC and chronic hepatitis in that the former but not the latter disease is associated with a defect in the spontaneous cytotoxic function of lymphocytes which lack surface immunoglobulin but bear Fc receptors.

4) Starting with peripheral blood mononuclear cells, depletion of complement receptor-bearing cells is associated with enrichment of Fc positive and surface immunoglobulin negative cells, depletion of phagocytes and surface immunoglobulin positive cells and normal or enhanced SCMC, MICC and ADCC. In contrast, depletion of Fc positive cells is associated with enrichment of complement receptor-bearing cells and virtually no capacity to induce SCMC or ADCC. These findings indicate that at least the majority of cells which mediate SCMC, MICC and ADCC do not bear complement receptors.

5) The clearance of both tracer and test doses of microaggregated albumin is normal in patients with compensated PBC, alcoholic cirrhosis and HBsAg negative chronic hepatitis, indicating normal hepatic blood flow and a normal non-specific phagocytic capacity of the reticuloendothelial system. The rate of clearance of IgG-coated radiochromated autologous red cells is normal in patients with compensated HBsAg negative chronic hepatitis and alcoholic cirrhosis. However, in the majority of patients with PBC, the rate of clearance of these cells was increased. This observation may imply activation of the reticuloendothelial system in this disease. In contrast, the clearance of C3b-coated radiochromated autologous red cells is markedly decreased in patients with compensated PBC, but is normal in patients with compensated HBsAg negative chronic hepatitis, alcoholic cirrhosis and large duct biliary obstruction. To obtain further insight into this clearance defect in PBC, a compartmental model of the C3b receptor-mediated clearance process has been developed. Using computer simulations the model is able to account for the observed data and shows that the major determinant of the defect is a decreased rate of deposition of C3b-coated cells on Kupffer cell receptors. The defect cannot be attributed to cholestasis, to deficiency of components of the classical complement pathway, or to increased activity of C3b-inactivator. However, the defect may be due to a decrease in the number or affinity of C3b-receptors or to partial saturation of these receptors by excess circulating C3b or immune complexes containing C3b.

6) Cultures containing unirradiated T cells and B cells from normal individuals show augmentation of immunoglobulin (Ig) synthesis at low T:B cell ratios (helper T cell effect) and inhibition of Ig synthesis at high T:B cell ratios (suppressor T cell effect). Cultures containing irradiated T cells from normal individuals show augmentation of Ig synthesis at all T:B cell ratios studied, indicating marked reduction of suppressor T cell function by irradiation. In contrast, cultures containing unirradiated T cells and autologous B cells from patients with PBC do not show inhibition of Ig synthesis at high T:B cell ratios, but instead show progressive increases in Ig synthesis with increasing T:B cell ratios up to the highest T:B cell ratio studied (10:1). Ig synthesis by cultures containing irradiated T cells and autologus B cells from patients with PBC is similar to that by corresponding cultures of cells from normal subjects. Allogeneic mixtures of cells from

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normal individuals and patients with PBC show that, while B cells from the majority of patients with PBC synthesize normal amounts of Ig in the presence of normal T cells, T cells from patients with PBC cause marked enhancement of Ig synthesis by normal B cells. Patients with other liver diseases characterized by chronic cholestasis, cirrhosis, or hepatocellular necrosis do not exhibit an analogous abnormality of Ig synthesis in vitro to that found in PBC. Thus, PBC is associated with a defect of immune regulation characterized by abnormally high Ig synthesis by cultures of autologous T and B cells at high T:B cell ratios. This defect appears to be due to a deficiency of suppressor T cell function.

7) Patients with PBC have a marked deficiency in the ability to generate the autologous mixed lymphocyte reaction but have a normal ability to generate an allogeneic mixed lymphocyte reaction. The defect in the autologous mixed lymphocyte reaction is not due to a serum factor. The deficiencies of both suppressor T cell function and the autologous mixed lymphocyte reaction in PBC could be due to a common defect of cell interactions involved in the generation of suppressor effector cells. Such a defect could play an important role in the pathogenesis of autoimmune disease.

Significance to Biomedical Research:

Eludication of the pathogenic mechanisms which initiate and sustain the disease processes of chronic active hepatitis and PBC is required for the development of more rational therapies. Defined initiating factors exist for those subgroups of chronic active hepatitis which are associated with HBsAg and which follow the ingestion of certain drugs, whereas initiating factors are unknown for most of the remainder of patients with chronic active hepatitis, (i.e. patients with lupoid hepatitis) and for all patients with PBC. The processes leading to sustained disease activity are unknown in both conditions. Current hypotheses favor immunologic mechanism(s). These hypotheses, in part, stem from the high prevalence of abnormal immunologic phenomena in both diseases. The empiric use of adrenal corticosteroids and other immunosuppressive therapies in these diseases appears to be, at least in part, a reflection of this view. To date, existing specific hypotheses relating to the pathogenesis of these diseases lack experimental confirmation. In addition, it is not known whether certain immunologic abnormalities found in some subgroups of patients and not in others are epiphenomena or are related to pathogenesis.

The current studies are designed to provide a comprehensive immunologic evaluation of selected patients so that the spectrum of immunologic abnormalities associated with PBC and HBsAg negative lupoid chronic hepatitis can be defined and the relationship of these abnormalities to clinical and histologic variables can be assessed. In addition, hypotheses of specific pathogenic mechanisms are tested. The significant morbidity and mortality of chronic active hepatitis, the uniform mortality of PBC, and the inadequacies of all current therapies in both conditions emphasize the need for information which throws light on the pathogenesis of these diseases.

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Proposed Course:

We will continue to apply up-to-date sophisticated immunologic techniques to define in greater depth the nature and significance of the immunologic defects exhibited by patients with HBsAg negative chronic active hepatitis and PBC.

Publications:

1. Lawley, T.J., James, S.P., Jones, E.A.: Circulating immune complexes: Their detection and potential significance in some hepatobiliary and intestinal diseases. Gastroenterology 78: 626-641, 1980.

2. James, S.P., Elson, C.O., Jones, E.A., Strober, W.: Abnormal regulation of immunoglobulin synthesis in vitro in primary biliary cirrhosis. Gastroenterology 79: 242-254, 1980.

3. Jones, E.A., James, S.P., Vierling, J.M.: Immunologic function in primary biliary cirrhosis: Defects recently demonstrated at the National Institutes of Health. In Chalmers, T.C. and Berk, P.D. (Eds.): Frontiers in Liver Diseases. New York, Stratton Intercontinental, 1980 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,504-06 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies of Copper Metabolism in Man Using ^{67}Cu and ^{64}Cu

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
Other:	R. Aamodt	Senior Radiobiologist	NM:CC
	W. Rumble	Biologist/Radiobiologist	NM:CC
	R. Shrager	Mathematician	LSMM:CR

COOPERATING UNITS (if any)
Nuclear Medicine Department, CC
Laboratory of Statistical and Mathematical Methodology, CR

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is currently inactive.

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,505-05 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of the Metabolism of Alpha-1-Antitrypsin and of Protease Inhibitor Phenotypes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
Other:	J. Vergalla	Chemist	DDB:NIAMDD
	J. Karsh	Guest Worker	A&R:NIAMDD
	J. E. Gadek	Clinical Associate	CB:NHLBI
	R. G. Crystal	Head, Pulmonary Branch	PB:NHLBI

COOPERATING UNITS (if any)

Pulmonary Branch, NHLBI

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/4	1/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Isolated preparations of α-1-antitrypsin (α1AT), trace-labeled with radioiodine, have been used to obtain data on the turnover of α1AT in normal subjects and patients with α1AT deficiency who have either pulmonary emphysema or chronic hepatocellular disease. The protease inhibitor (Pi) phenotypes of subjects donating plasma for isolation of α1AT and subjects undergoing α1AT turnover studies have been determined. The fractional catabolic rates of M α1AT from normal subjects (phenotype PiMM) and Z α1AT from patients with α1AT deficiency (phenotype PiZZ) are similar in both normal subjects and patients with α1AT deficiency. These findings imply that the low serum α1AT concentration in α1AT deficiency is due to impaired hepatic release of α1AT and that the differences in molecular structure between the Z and M proteins have little effect on the catabolism of α1AT. In common with other glycoproteins, desialylated (i.e. neuraminidase-treated) α1AT is rapidly cleared from plasma by the liver. Pi phenotypes have been determined in populations of patients with rheumatoid arthritis, systemic lupus erythematosis and Sjogren's syndrome.

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Project Description:

Objectives:

Alpha-1-antitrypsin (α1AT) is a plasma glycoprotein which contains sialic acid and is responsible for most of the tryptic inhibitory capacity of plasma. The objectives of this study are to obtain data on the metabolism of α1AT in normal subjects (protease inhibitor [Pi] phenotype MM), patients with α1AT deficiency (PiZZ), relatives of PiZZ individuals with one Z gene and patients with hepatocellular disease unassociated with α1AT deficiency. The possibility that the degree of sialylation of this protein, which is less in PiZZ than PiMM individuals, influences its metabolism in vivo will be investigated by comparing the metabolism of α1AT's isolated from PiMM and PiZZ individuals in the same subjects and by studying the metabolism of α1AT which has been desialylated in vitro. The necessity to perform Pi phenotyping for these studies permits additional studies in which the distribution of Pi phenotypes in selected populations is determined.

Methods Employed:

1) The Pi phenotypes of subjects donating plasma for isolation of α1AT and subjects undergoing studies of α1AT metabolism are determined using acid starch gel electrophoresis followed by crossed immunoelectrophoresis and/or slab gel isoelectric focusing in acrylamide.

2) α1AT is isolated from plasma using $(NH_4)_2SO_4$ precipitation, gel exclusion chromatography, Concanavalin A affinity chromatography, DEAE ion exchange chromatography and affinity chromatography using blue dextran and activated thiol exchange.

3) The purity of isolated α1AT is determined using crossed immunoelectrophoresis, polyacrylamide gel electrophoresis and isoelectric focusing. Its biological activity is assessed by a kinetic assay of tryptic inhibitory capacity using an artificial substrate.

4) To quantitate the turnover of α1AT in man, pure biologically active α1AT trace labeled with radioiodine is administered intravenously and serial measurements of radioactivity in the whole body, plasma and urine are analyzed.

Major Findings:

1) Isolated (PiMM) α1AT has been shown to be immunochemically pure and to retain both its characteristic electrophoretic microheterogeneity on acid starch gel electrophoresis and its ability to inhibit trypsin.

2) The half life of radioiodinated human (PiMM) α1AT in the plasma of rabbits varied from 8.7 to 10.4 hours whereas that of the same protein after desialylation was less than 10 minutes.

3) Ranges for pool sizes, transfer rates between pools and synthetic and fractional catabolic rates of α1AT in normal subjects have been determined.

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4) In turnover studies of MM αlAT in normal (PiMM) volunteers, the serum concentration of αlAT was shown to vary independently of its fractional catabolic rate suggesting that a constant volume of plasma is cleared of α_1AT per unit time. Analysis of the turnover data indicated that α_1AT is catabolized in, or in close relationship to, the intravascular compartment.

5) In analogous studies in normal volunteers the desialylated protein was shown to be removed from plasma approximately 150 times faster than the intact protein. Its rapid disappearance from plasma was associated with an equally rapid uptake of the radioiodine label by the liver. This finding emphasizes that the catabolism of αlAT can be influenced by the molecular structure of the carbohydrate moiety and suggests that hepatic receptors for desialylated glycoproteins exist in the human liver.

6) A patient with the rare Pi phenotype SZ, αlAT deficiency and cirrhosis associated with globules of αlAT material in his hepatocytes was shown to have appreciable hepatic copper overload, chronic fibrosing interstitial pneumonitis and, terminally, a hepatocellular carcinoma.

7) The fractional catabolic rate of the Z protein in patients with αlAT deficiency is similar to that of the M protein in normal subjects. Furthermore, the fractional catabolic rates of the M protein in patients with αlAT deficiency and of the Z protein in normal subjects are similar to that of the M protein in normal subjects. Thus, the low serum concentration of αlAT in αlAT deficiency is due to impaired hepatic release of αlAT rather than hypercatabolism of αlAT. Futhermore, differences in the molecular structure of the Z and M proteins do not affect the catabolism of αlAT to any appreciable extent.

8) No significant increase in non-MM Pi phenotypes was found in 37 patients with rheumatoid arthritis and 40 patients with systemic lupus erythematosus suggesting that particular Pi types neither predispose to the development nor enhance the severity of these diseases.

9) No significant increase in non-MM Pi phenotypes was found in 52 patients with Sjogrens syndrome. Mean values for tests of pulmonary function, including estimates of both restrictive lung disease and airway obstruction were not significantly different for patients with Sjogren's syndrome who had the Pi MM phenotype and those with non-MM Pi phenotypes. Thus, patients with Sjogren's syndrome who have non-MM Pi phenotypes (partial αlAT deficiency) do not appear to have an increased susceptibility to develop chronic obstructive pulmonary disease.

Significance to Biomedical Research:

Homozygous αlAT deficiency (phenotype PiZZ) may be associated with cholestasis in the neonatal period, cirrhosis, liver cell carcinoma and emphysema at a young age. In this condition, hepatocytes have been shown to contain globules of αlAT-like material which is devoid of sialic acid, and the αlAT in plasma, which is present in abnormally low concentration, has also been shown to have a reduced content of sialic acid. The relationship

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of the characteristic hepatic globules and the low plasma concentration of αlAT to the hepatic lesions associated with αlAT deficiency is unknown. This study is designed to investigate the metabolism of αlAT in αlAT deficiency. The relationship of the characteristic hepatic globules to the rate at which αlAT is delivered to plasma and to the concentration of αlAT in plasma is assessed. In addition, the contribution of increased catabolism or hepatic uptake of αlAT to reduced plasma concentrations of αlAT is determined. The study determines the effect of certain differences in the molecular structure of αlAT (e.g. different degrees of sialylation) on its rate of endogenous catabolism and hepatic uptake. In particular, the hypothesis that there are hepatic receptors for exposed galactose residues on desialylated glycoproteins which facilitate the rapid hepatic clearance of these proteins is tested in man.

The observation that desialylated glycoproteins appear to be metabolized by hepatic lysosomes suggests that measurements of hepatic radioactivity after administering a radioiodinated desialylated glycoprotein such as αlAT could be used as a test for hepatic lysosomal function.

The determination of Pi phenotypes enables the frequency distribution of this genetic marker to be determined in well-defined populations of patients.

Proposed Course:

The studies in progress will be completed. The frequency distribution of of Pi phenotypes will be determined in sera from selected well-defined populations of patients.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 53,506-04 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of the Properties of Desialylated Human Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.:	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
Other:	C. J. Steer	Clinical Associate	DDB:NIAMDD
	S. P. James	Clinical Associate	DDB:NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Desialylated (i.e. neuraminidase-treated) lymphocytes (DL) induce significantly more cytotoxicity in isolated rabbit hepatocytes than do intact lymphocytes. This phenomenon can be selectively blocked by a desialylated glycoprotein and is attributed to the receptor for desialylated glycoproteins (hepatic binding protein, HBP) on the surface of hepatocytes interacting with its specific ligands on the surface of DL. Purified HBP increases the cytotoxicity of DL to target cells which do not bear HBP on their surface presumably by an analogous mechanism. Infused desialylated peripheral blood mononuclear cells (PBMC) and DL are selectively taken up by the liver in rabbits. The phenomenon is unaffected by depletion of complement but can be blocked by preincubation of desialylated PBMC with IgG-Fab fragments. These findings suggest that the selective hepatic uptake of desialylated PBMC or DL is not mediated by HBP. They are compatible with desialylated PBMC or DL becoming coated with naturally occurring antibodies in vivo with the result that subsequent recognition of cell bound antibody by Fc receptors on Kupffer cells mediates their selective hepatic uptake.

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Project Description:

Objectives:

Hepatic binding protein (HBP), a normal constituent of the surface membrane of mammalian hepatocytes, can induce mitogenesis in desialylated lymphocytes. This project is designed to determine whether HBP has additional immunologic properties such as the ability to induce desialylated lymphocytes to become cytotoxic, that is to mediate mitogen-induced cellular cytotoxicity (MICC). Desialylated lymphocytes are selectively taken up by the liver. Studies are designed to determine the probable mechanism of this phenomenon, including the role, if any, of HBP.

Methods Employed:

HBP is isolated in a pure form from rabbit liver using affinity chromatography. HBP in the surface membranes of hepatocytes is measured by its ability to bind ^{125}I-asialo-orosomucoid. For assays of cytotoxicity lymphocytes are isolated from the blood of normal human volunteers by centrifugation over Ficoll-Hypaque. Lymphocytes are desialylated by incubating with neuraminidase. Two in vitro assays of cytotoxicity are used. One measures the release of radioactivity from ^{51}Cr-labeled nucleated target cells after their incubation with effector cells in the presence or absence of mitogens. The other measures the loss of ability of target cells to adhere to plastic after incubation with effector cells. Viability of both effector and target cells is measured by the ability of cells to exclude the dye trypan blue. Chang cells are used as nonspecific nucleated target cells. Pure rabbit hepatocytes, isolated by perfusing the liver in situ with a solution containing collagenase and hyaluronidase, are used as hepatospecific target cells containing exposed HBP on their surface membrane.

After the infusion of ^{51}Cr-labeled desialylated and normal peripheral blood mononuclear cells (PBMC) and lymphocytes into rabbits the fate of labeled cells is determined from measurements of radioactivity in plasma, liver and other organs. The effects of complement depletion using Cobra venom factor and preincubation with IgG-Fab fragments on the hepatic uptake of labeled desialylated PBMC are quantitated.

Major Findings:

1. Cytotoxicity of desialylated lymphocytes against nonspecific nucleated target cells in the presence of HBP is equivalent to that in the presence of the powerful plant mitogen phytohemagglutinin and is much greater than that induced by these cells in the absence of HBP. Having shown that isolated rabbit hepatocytes contain exposed HBP in their surface membrane, it was found that a much greater proportion of these cells lose their ability to adhere to plastic after incubation with desialylated lymphocytes than after incubation with normal untreated lymphocytes. This effect of desialylated lymphocytes was specifically inhibited by asialo-orosomucoid, a desialylated glycoprotein, but not by intact orosomucoid. These findings indicate that HBP can mediate MICC not only as a purified protein but also as a constituent of the surface membrane of a mammalian hepatocyte.

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2. The selective hepatic uptake of the label of ^{51}Cr-labeled desialylated peripheral blood mononuclear cells (PBMC) and desialylated lymphocytes has been demonstrated in the rabbit. This phenonenon does not appear to be due to the specific interaction between exposed galactose residues on the surface of the desialylated cells and HBP which specifically recognizes exposed galactose residues on desialylated glycoproteins, since sinusoidal cells, which come into contact with circulating desialylated cells do not bind desialylated glycoproteins and hence do not bear HBP. Consistent with this reasoning is the observation that after infusions of desialylated lymphocytes into rabbits neither serial values for serum transaminases nor hepatic histology were indicative of hepatocellular injury. Naturally occurring antibodies exist in normal plasma which specifically recognize antigens exposed on the surface of lymphocytes by desialylation. These antibodies probably do not mediate the selective hepatic uptake of desialylated cells through the action of complement as complement depletion of rabbits in vivo was without effect on the hepatic uptake of the label of ^{51}Cr-labeled desialylated cells. However, preincubation of labeled desialylated PBMC with rabbit IgG Fab fragments reduced the hepatic uptake of the label of these cells to that of the label of ^{51}Cr-labeled intact PBMC, suggesting that the Fab fragments were able to bind to exposed antigens on desialylated cells and inhibit binding of circulating antibodies to these antigens. These findings are compatible with the hypothesis that desialylated PBMC or lymphocytes in vivo become coated with naturally occurring antibodies with subsequent recognition of cell-bound antibody by Fc receptors on Kupffer cells mediating their selective hepatic uptake.

Significance to Biomedical Research:

For the first time MICC has been shown to be mediated by a normal constituent of the surface membrane of a mammalian cell. The results of these studies demonstrate that a minor structural change in the surface membrane of lymphocytes might result in their becoming cytopathogenic when exposed to normal hepatocytes. Thus MICC may be a mechanism of hepatocellular injury in vivo. This observation raises the possibility that interaction between a variety of constituents of normal cell membranes and lymphocytes whose surface membranes have undergone subtle structural changes may be a mechanism of immune-mediated cellular injury in human disease.

The clearance of desialylated lymphocytes by Fc receptors on Kupffer cells in vivo may prevent the mediation of hepatocellular injury by the interaction of desialylated lymphocytes with HBP on the surface of hepatocytes. This may be particularly significant since the enzyme responsible for desialylation, neuraminidase, appears to be widely distributed in vivo and occurs in organisms pathogenic to man such as the influenza virus. The coating of desialylated cells by naturally occurring antibodies in vivo and their subsequent accelerated clearance from the circulation by Fc receptors on Kupffer cells may be a general phenomenon which applies to all formed elements of the blood including platelets. For instance, it may be a physiologic mechanism for the removal of senescent red cells from the circulation as the surface membrane of such cells contains much less sialic acid than the surface membrane of young red cells.

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Z01 AM 53,506-04 DDB

Proposed Course:

No further studies are planned.

Publications:

1. Steer, C.J., James, S.P., Vierling, J.M., Hosea, S.W., Jones, E.A.: The selective hepatic uptake of desialylated peripheral blood mononuclear cells in rabbits. Gastroenterology, 1980 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,507-03 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Synthesis and Binding of Collagen by Hepatocytes in Chronic Liver Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	J. G. Waggoner	Chemist	DDB:NIAMDD
	H. K. Kleinman	Senior Staff Fellow	LDBA:NIDR
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COOPERATING UNITS (if any)
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LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/4	0	1/4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interaction between hepatocytes and collagen may be of importance in the pathogenesis of chronic hepatocellular diseases. The adherence of each of the five types of collagen to hepatocytes isolated from normal rats and rats with carbon tetrachloride-induced hepatic fibrosis has been studied. Attachment of hepatocytes from normal rats to collagen was enchanced four-fold by the presence of either serum or fibronectin, the glycoprotein required for fibroblast attachment to collagen. In the absence of serum or fibronectin, hepatocytes adhered best to basement membrane collagen (type IV). This phenomenon was abolished by sodium periodate oxidation of collagen, suggesting that hepatocytes attached to collagen by recognizing a specific carbohydrate residue. Hepatocytes isolated from rats with hepatic fibrosis bound less avidly to collagen than those from normal rats. Neither hepatocytes from normal rats nor those from rats with hepatic fibrosis appear to synthesize collagen in vitro. These studies indicate a defect in the interaction between hepatocytes and collagen in an animal model of chronic liver disease.

Project completed.

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Project Description:

Objectives:

It is proposed to determine whether the interaction between hepatocytes and collagen may be abnormal in chronic hepatocellular disease. The ultimate objectives of this project is to define the factors responsible for the laying down of increased quantities of collagen in the liver in chronic liver diseases and to determine how the progression of hepatic fibrosis might be inhibited or prevented.

Methods Employed:

1) The induction of hepatic fibrosis in rats using carbon tetrachloride.

2) The preparation of suspensions of cells from rat livers perfused with a solution containing collagenase and the subsequent isolation of pure preparations of hepatocytes by density sedimentation.

3) Cultures of hepatocytes with different types of collagen.

4) Immunofluorescent staining of cells with antibodies specific for different procollagens and immunofluorescence microscopy.

5) Measurements of the incorporation of the label of ^{3}H-proline into proteins synthesized by hepatocytes.

Major Findings:

1) Attachment of hepatocytes from normal rats to collagen was enhanced four-fold by the presence of serum or fibronectin, the glycoprotein required for fibroblast attachment to collagen.

2) In the absence of fibronectin hepatocytes adhered best to basement membrane collagen (type IV).

3) Periodate oxidation of collagen abolishes its binding to hepatocytes suggesting that the carbohydrate moiety of collagen may be involved in the binding.

4) Hepatocytes isolated from rats with hepatic fibrosis bind to collagen less avidly that do those from normal rats.

5) Procollagens were not demonstrated immunofluorescently in hepatocytes but were found in cells with fibroblastic morphology.

6) Hepatocytes from normal rats and from rats with hepatic fibrosis appear to synthesize similar amounts of collagen.

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Significance to Biomedical Research:

Hepatic fibrosis is a major component of the disturbed hepatic architecture in most chronic liver diseases. In cirrhosis it is associated with the development of portal hypertension and episodes of bleeding into the gastrointestinal tract from esophageal varices. This project is designed to investigate the pathogenesis of the abnormal laying down of collagen and fibrosis in the liver in chronic liver disease. The results obtained may suggest means of decreasing the rate of progression of hepatic fibrosis or preventing it altogether.

Proposed Course:

Data from recent studies have been analyzed and published. No further studies are planned at this time.

Publication:

1. Berman, M.D., Waggoner, J.G., Foidart, M.J., Kleinman, H.K.: Attachment to collagen by isolated hepatocytes from rats with induced hepatic fibrosis. J. Lab. Clin. Med. 95: 660-671, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,508-03 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of Hepatic Receptors for Modified Glycoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
Other:	C. J. Steer	Clinical Associate	DDB:NIAMDD
	S. Furbish	Senior Staff Fellow	DMNB:NINCDS
	J. A. Barranger	Chief, Clinical Section	DMNB:NINCDS
	R. O. Brady	Chief, Dev. Met. Neur. Br.	DMNB:NINCDS
	R. Clarenberg	Guest Worker	LBM:NIAMDD
	G. Ashwell	Chief, Lab. Biochem. and Metab.	LBM:NIAMDD

COOPERATING UNITS (if any)
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Development and Metabolic Neurology Branch, NINCDS

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The binding of radiolabeled modified glycoproteins to parenchymal and non-parenchymal cell preparations isolated from the perfused rat liver has been studied. Hepatocytes possess receptors for glycoproteins with galactose as the terminal sugar residue. Non-parenchymal cells lack a receptor for galactose-terminated glycoproteins but specifically bind N-acetylglucosamine and mannose-terminated glycoproteins. The effect of modifying the carbohydrate moiety of glucocerebrosidase on the distribution of its cellular uptake after intravenous infusion into rats was studied. The carbohydrate moiety of this enzyme could be modified without loss of enzyme activity. Galactose-terminated glucocerebrosidase was selectively taken up by hepatocytes. Exposure of N-acetylglucosamine or mannose residues markedly augmented the uptake of the enzyme by non-parenchymal cells. The ability to manipulate the cellular distribution of an infused enzyme may be of importance in enzyme replacement therapy. The intact glycoprotein, human β-hexosaminidase A, is rapidly cleared from the circulation by a mannose-specific recognition system on hepatic sinusoidal cells. This process may be of physiologic importance in preventing the accumulation of potentially autodestructive lysosomal enzymes in the circulation.

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Project Description:

Objectives:

This study was designed to determine whether receptors for modified glycoproteins, other than the receptor previously described for galactose-terminated glycoproteins (HBP), exist in the mammalian liver. It was also designed to assess the distribution of receptors for modified glycoproteins, including HBP, between parenchymal and non-parenchymal hepatic cells. The potential implications of these receptors to clinical medicine have been the subject of additional studies. In particular, the effects of modifying the carbohydrate moiety of glucocerebrosidase, the enzyme deficient in Gaucher's disease, on its cellular distribution within the liver after infusion have been determined. The cell type responsible for the rapid hepatic clearance of a lysosomal enzyme (β-hexosamindase A) and the glycosyl specificity of this process has also been investigated.

Using isolated hepatocytes the intracellular:cell surface membrane distribution ratio of HBP, the stability of HBP and the ability of HBP to recycle between the cell membrane and intracellular structures have been studied.

Methods Employed:

1) Suspensions of hepatic cells are obtained from rat liver after in situ perfusion with a solution of collagenase. Parenchymal and non-parenchymal hepatic cells are subsequently isolated from cell suspensions using gravity sedimentation and Metrizamide density gradient centrifugation.

2) For binding studies, the carbohydrate moiety of native glycoproteins is modified by incubations with appropriate enzymes (neuraminidase, β-galactosidase, N-acetylglucosaminidase).

3) Glucocerebrosidase activity is assayed in detergent buffered extracts of sonicated cells.

4) Glycoproteins are radioiodinated using Enzymobeads and the Chloramine-T method.

5) Standard assay techniques are employed to determine the uptake of labeled ligands by cells incubated in vitro. The amount of unlabeled ligand mixed with the appropriate labeled ligand is varied.

6) To study the kinetics of binding of ^{125}I-labeled galactose terminated glycoproteins to HBP, isolated hepatocytes are maintained in suspension. Cycloheximide is used to inhibit protein synthesis by such cells. Cells are subjected to detergent solubilization to determine the total amount of HBP in hepatocytes (intracellular and surface membrane).

Major Findings:

1) Significant binding of galactose, but neither N-acetylglucosamine

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nor mannose-terminated orosomucoid to intact hepatocytes was demonstrated. Non-parenchymal cells did not bind galactose-terminated orosomucoid but bound significant amounts of N-acetylglucosamine and mannose-terminated orosomucoid. Thus, Kupffer cells lack a receptor for galactose-terminated glycoproteins. Carrier dilution studies indicated the presence of a distinct receptor for galactose-terminated glycoproteins on hepatocytes and a distinct receptor with mannose specificity on non-parenchymal cells.

2) The carbohydrate moiety of the enzyme glucocerebrosidase can be modified without the glycoprotein losing enzyme activity. Galactose-terminated glucocerebrosidase is preferentially taken up by hepatocytes. Exposure of N-acetylglucosamine or mannose sugar residues markedly augments the uptake of glucocerebrosidase by non-parenchymal hepatic cells.

3) The rapid in vivo hepatic uptake of the glycoprotein β-hexosaminidase A exhibits mannose specificity. In vitro no uptake of this enzyme by hepatocytes is detectable, whereas there is appreciable uptake of the enzyme by sinusoidal cells. This uptake process also exhibits mannose specificity. The kinetics of uptake of β-hexosaminidase A and mannose-terminated orosomucoid by sinusoidal cells are similar.

4) Of the total HBP in rat hepatocytes 5% is on the cell surface and 95% is intracellular. The average time an individual HBP receptor remains on the cell surface is 3 minutes. More ^{125}I-labeled galactose-terminated glycoprotein is metabolized in hepatocytes per unit time than can be bound by the total number of intracellular and surface membrane HBP receptors under conditions of inhibition of protein synthesis. This finding indicates that HBP exhibits no down regulation and suggests that the receptor is either recycled or is re-utilized at its plasma membrane site.

Significance to Biomedical Research:

Hepatic receptors for glycoproteins with different terminal sugar residues may be of importance in the catabolism of glycoproteins and in the hepatic uptake of cells with modified glycoproteins on their surface membrane. The ability to manipulate the cellular distribution of an infused glycoprotein enzyme by modifying its carbohydrate moiety may be of clinical importance in enzyme replacement therapy. For instance, in Gaucher's disease, in which there is a deficiency of the enzyme glucocerebrosidase, glucocerebroside accumulates in Kupffer cells. Increased uptake of infused glucocerebrosidase by Kupffer cells may be achieved by exposing mannose as the terminal sugar of its carbohydrate moiety. Receptor mediated hepatic clearance of lysosomal enzymes, such as β-hexosaminidase A, may be of physiologic importance in preventing the accumulation in the circulation of potentially autodestructive enzymes. Studies of the kinetics of the interactions of HBP with its specific ligands provide important insights into the properties of receptors in general, e.g. the recycling of receptors.

Proposed Course:

No further studies are proposed at this time.

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MAY BE VIOLATED UP TO 2 CHARACTERS.

Publications:

1. Steer, C.J., Ashwell, G.: Studies on a mammalian hepatic binding protein specific for asialoglycoproteins: Evidence for receptor recycling in isolated rat hepatocytes. J. Biol. Chem. 255: 3008-3013, 1980.

2. Jones, E.A., Vierling, J.M., Steer, C.J.: Surface receptors on hepatic sinusoidal cells. In Bianchi, L., Popper, H. (Eds.): Communication of Liver Cells. Lancaster, England, MTP Press Limited, 1980 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 53,509-02 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of patients who are chronic carriers of hepatitis B surface antigen (HBsAg)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	J. H. Hoofnagle	Senior Investigator	DDB:NIAMDD
Other:	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
	S. P. James	Clinical Associate	DDB:NIAMDD
	D. F. Schafer	Clinical Associate	DDB:NIAMDD
	R. H. Purcell	Chief, Hepatitis Section	LID:NIAID
	S. M. Feinstone	Senior Investigator	LID:NIAID
	J. Gerin	Head, Molec. Virol. & Immunol. Div.	Georgetown University
	R. Johnson	Medical Officer	LID:NIAID
	S. E. Straus	Senior Investigator	LCI:NIAID

COOPERATING UNITS (if any)
LID and LCI, NIAID
Georgetown University School of Medicine

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1	1/2

CHECK APPROPRIATE BOX(ES)

[X] (a) HUMAN SUBJECTS [X] (b) HUMAN TISSUES [ ] (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Persons with HBsAg-positive chronic hepatitis are being enrolled in a prospective study of this disease. Thirty persons have been entered into this study, 22 of whom have been followed for at least 1 year (and 8 for 2 years). 20 patients have had liver biopsies. At the start all except one patient had elevated serum aminotransferase levels and all were HBsAg positive; 27 had HBeAg and 24 DNA-polymerase detectable in serum. On follow-up, one has lost HBsAg but 8 have lost HBeAg. These 8 who seroconverted from HBeAg to anti-HBe have also had a spontaneous clinical remission with disappearance of all symptoms and a return of serum aminotransferase levels to normal. The remaining patients, who are persistently HBeAg-positive, have had stable levels of HBsAg, HBeAg and aminotransferases. In individuals with stable hepatitis B virus markers, trials of antiviral agents are being planned. Initially 10-15 persons are being treated with adenine arabinoside monophosphate (Ara-AMP) . Because there is a natural spontaneous rate of remssion in this disease a controlled clinical trial will be planned in the event that Ara-AMP seems to be effective against the hepatitis B virus.

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Project Description:

Objectives:

1) This study is designed to establish a group of patients with well characterized chronic HBsAg-positive hepatitis in preparation for treatment trials with antiviral agents.

2) Experiments are also designed to determine if subjects in whom HBsAg persists after hepatitis B infection have specific immunologic defects, such as an impaired ability to synthesize anti-HBs.

Methods Employed:

1) Radioimmunoassays have been established for detection and quantification of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), antibody to HBsAg (anti-HBs), antibody to hepatitis B core antigen (anti-HBc) and antibody to HBeAg (anti-HBe). 2) Separation of IgG from IgM classes of antibodies using sucrose density gradient ultracentrifugation analysis. 3) An enzymatic assay for DNA-polymerase. 4) Liver biopsies are processed for light, immunofluorescence and electron microscopy. 5) Immunologic studies of patients with chronic type B hepatitis are also being conducted. Specific immunity to various hepatitis B virus agents (HBsAg, HBeAg, HBcAg) is being evaluated by means of antigen-induced lymphocyte proliferation assays and by assays for *in vitro* production of specific antibody by cultured stimulated lymphocyte populations.

Major Findings:

Thirty HBsAg-positive persons have been enrolled in this project. Twenty-two have been followed for at least one year and 8 for at least two years. Only one of the thirty patients has lost HBsAg-reactivity during this time. HBsAg was initially present in serum in amounts ranging from 4-500 mcg/ml. Levels of HBsAg have been very stable in most of the patients. However, in six of 22 patients followed for one year, titers of HBsAg have fallen by at least four-fold. Initially 27 patients were positive for HBeAg and 24 for hepatitis B virus specific DNA-polymerase activity. During follow-up of at least one year, eight patients have lost HBeAg reactivity and DNA-polymerase activity. Most striking, however, was that all eight of these patients underwent a spontaneous remission of all clinical symptoms and of serum biochemical evidence of chronic hepatitis. Thus, serum aminotransferases were elevated in all HBeAg-positive persons when first evaluated. During follow-up evaluation, serum aminotransferase levels fell to normal values in the eight persons who lost HBeAg and DNA-polymerase reactivity.

Percutaneous liver biopsies have been obtained from 20 of the 30 patients. Light microscopy appearances of the biopsies did not correlate with eventual outcome, i.e. spontaneous remission or persistence of disease activity.

Significance to Biomedical Research:

The goal of this project is to investigate the natural history of chronic HBsAg positive hepatitis, and to execute appropriately designed trials of

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anti-viral agents in this disease. As shown, there is a spontaneous remission rate for this disease of approximately 15-25% each year. Obviously, trials of antiviral agents will need to include sufficient numbers of treated and untreated persons to ensure that the effect of treatment is to induce a remission rate which is greater than the spontaneous remission rate for this disease. Studies such as this will also help to identify which patients are likely to benefit most from therapy. Patients currently considered most suitable for therapy with antiviral agents are those who have stable levels of hepatitis B virus markers - HBeAg, HBsAg and DNA-polymerase - and who have rapidly progressive chronic active hepatitis as demonstrated by serial percutaneous liver biopsies.

Proposed Course:

We plan to continue to follow the 30 patients already admitted to this study and to follow 20-30 additional similar patients in order to define more precisely the spontaneous remission rate of this disease and to identify serological and virological markers of prognostic value. Patients who exhibit stable levels of HBeAg, HBsAg and DNA-polymerase will be entered into therapeutic trials of anti-viral agents. As part of a pilot study, seven such patients have agreed to receive the anti-viral agent adenine arabinoside monophosphate (Ara-AMP) (protocol no. 80-A-I-53). Based upon the results of the pilot study (which is designed to evaluate dosage, toxicity and potential therapeutic effects of the drug), a randomized controlled trial of Ara-AMP in the treatment of chronic HBsAg positive hepatitis may be initiated.

Publications:

1. Tabor, E., Hoofnagle, J.H., Smallwood, L.A., Drucker, J.A., Pineda-Tamondong, G.C., Ni, L.Y., Greenwalt, T.J., Barker, L.F., Gerety, R.J.: Studies of donors who transmit post-transfusion hepatitis. Transfusion 19: 725-731, 1979.

2. Hoofnagle, J.H.: Current concepts in viral hepatitis. Arquivos de Gastroenterologica, Sao Paul 16: 124-132, 1979.

3. Hoofnagle, J.H., Waggoner, J.G.: Hepatitis A and B virus markers in immune serum globulin. Gastroenterology 78: 259-263, 1980.

4. Hoofnagle, J.H., Seeff, L.B., Bales, Z.B., Wright, E.C., Zimmerman, H.J., and the Veterans Administration Cooperative Study Group: Passive-active immunity from hepatitis B immune globulin. Reanalysis of a Veterans Administrative Cooperative Study of Needle-Stick Hepatitis. Ann. Intern. Med. 91: 813-818, 1979.

5. Seeff, L.B., Hoofnagle, J.H.: Immunoprophylaxis of viral hepatitis. Gastroenterology 77: 161-182, 1979.

IMPORTANT: (1) SINGLE SPACE, EXCEPT BETWEEN PARAGRAPHS.
(2) USE REASONABLY NEW BLACK TYPEWRITER RIBBON, PREFERABLY CARBON PAPER RIBBON.
(3) "OFFSET REPRODUCTION" RIBBON IS NOT NECESSARY OR DESIRABLE.

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IMPORTANT: (1) SINGLE SPACE, EXCEPT BETWEEN PARAGRAPHS.
(2) USE REASONABLY DRY BLACK TYPEWRITER RIBBON, PREFERABLY CARBON PAPER RIBBON.
(3) "OFFSET REPRODUCTION" RIBBON IS NOT NECESSARY OR DESIRABLE.

6. Gerety, R.J., Hoofnagle, J.H., Barker, L.F.: Hepatitis associated with hemophilia treatment. In Mammen, E.F., Barnhart, M.I., Lusher, J.M., Walsh, R.T. (Eds.): Treatment of Bleeding Disorders. New York, PJD Publications Limited, 1980, pp 199-215.

7. Hoofnagle, J.H.: Immunoprophylaxis of viral hepatitis. Ann. Intern. Med. 92: 870, 1980 (letter).

END →

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,510-01 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Study of the Natural History of Chronic Non-A, Non-B Hepatitis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	J. H. Hoofnagle	Senior Investigator	DDB:NIAMDD
Other:	H. J. Alter	Chief, Immunology Section	BB:CC
	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
	G. M. Dusheiko	Visiting Associate	DDB:NIAMDD
	D. F. Schafer	Clinical Associate	DDB:NIAMDD
	R. H. Purcell	Chief, Hepatitis Section	LID:NIAID
	H. Denes	Visiting Scientist	LID:NIAID
	S. M. Feinstone	Senior Investigator	LID:NIAID
	K. Ishak	Chief, Hepatic Pathology Branch	A.F.I.P.

COOPERATING UNITS (if any)
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Armed Forces Institute of Pathology

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with well-documented chronic non-A, non-B (non-lupoid) hepatitis are being evaluated to determine the natural history of this disease and to obtain tissues and reagents to search for non-A, non-B agents. Thirteen patients have been admitted to this study. These patients have undergone thorough evaluation including a liver biopsy in twelve. They have had the disease for periods ranging from 1 to 6 years. Four patients have a mild degree of malaise, the remaining patients are asymptomatic. Liver biopsies have revealed chronic persistent hepatitis in two and chronic active hepatitis in the rest. One patient has subsequently developed cirrhosis. Examination of liver biopsies by electron and immunofluorescence microscopy has failed to show definitive evidence of viral antigens or viral particles. However, electron microscopy of three biopsies has revealed intranuclear virus-particle-like structures which are similar to those observed in chimpanzees with non-A, non-B hepatitis. The significance and specificity of these structures is under investigation.

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MAY BE VIOLATED UP TO 2 OR 3 LETTERS.

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IMPORTANT: (1) SINGLE SPACE, EXCEPT BETWEEN PARAGRAPHS.
(2) USE REASONABLY NEW BLACK TYPEWRITER RIBBON, PREFERABLY CARBON PAPER RIBBON.
(3) "OFFSET REPRODUCTION" RIBBON IS NOT NECESSARY OR DESIRABLE.

Project Description:

Objectives: A third form of viral hepatitis - non-A, non-B - was first described in 1975. Since that time non-A, non-B hepatitis has been shown to be the most common type of post-transfusion hepatitis and to account for at least 20% of sporadic viral hepatitis. Non-A, non-B hepatitis from transfusion has a high propensity to progress to chronic hepatitis. The nature of the chronic hepatitis, its natural history, its therapy, its pathogenesis and its viral etiology remain poorly defined. The purpose of this study is to define the natural history to post-transfusion non-A, non-B hepatitis and to provide reagents (blood and liver biopsy tissue) for attempts to identify the agent(s) of non-A, non-B hepatitis.

Method Employed:

Patients followed in the post-transfusion hepatitis studies conducted by the NIH Blood Bank who develop non-A, non-B hepatitis and who have persistence of elevated serum aminotransferase levels (ALT, AST) for more than 6 months are evaluated by the Liver Diseases Section. Patients undergo a comprehensive medical evaluation. Blood studies include HLA typing and collection of serum for agar gel diffusion and counterelectrophoresis tests for non-A, non-B antigens. If indicated, a percutaneous liver biopsy is obtained and the biopsy subjected to light, immunofluorescence and electron microscopy.

Major Findings:

Thirteen patients have been evaluated and twelve have undergone percutaneous liver biopsy. Patients have had evidence of hepatitis for periods of time varying between 1 and 6 years. Four patients experience mild degrees of lethargy or malaise. The remaining patients have no symptoms attributable to liver disease. In contrast to chronic type B hepatitis serum aminotransferase levels fluctuate over a wide range, i.e. from normal values to as high as 700 I.U./l. So far, HLA typing has not revealed any correlations of note. In particular, the incidence of B8, DrW 3 types, which is increased in patients with autoimmune (lupoid) chronic hepatitis, is not increased in this population of patients with non-A, non-B hepatitis. Liver biopsies have revealed chronic active hepatitis in 10 patients and chronic persistent hepatitis in two. Follow-up liver biopsies from four patients, whose initial biopsies revealed chronic active hepatitis, showed cirrhosis in one and milder degrees of hepatocellular disease in the rest. One of these biopsies shows chronic persistent hepatitis.

Immunofluorescence studies on the liver biopsies using sera from patients who have recovered from non-A, non-B hepatitis have not revealed any potential non-A, non-B antigens. Furthermore, electron microscopy of the biopsies has not demonstrated viral antigens or particles in the liver biopsies. However, three biopsies have shown peculiar intranuclear clusters of pleomorphic particles. Similar structures have also been seen in liver biopsies from chimpanzees with non-A, non-B hepatitis. The significance and specificity of these nuclear changes are being investigated. Agar gel diffusion and counterelectrophoresis studies of sera from these patients have not demonstrated an antigen-antibody system that might be specific for this disease.

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IMPORTANT: (1) SINGLE SPACE, EXCEPT BETWEEN PARAGRAPHS.
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(3) "OFFSET REPRODUCTION" RIBBON IS NOT NECESSARY OR DESIRABLE.

Significance to Biomedical Research:

Non-A, non-B hepatitis is the most common form of post-transfusion hepatitis and occurs in 3-10% of transfused individuals. At present there are no markers for this disease and no associated viral antigens or particles that have been definitively identified. Chronic liver disease due to non-A, non-B hepatitis is also common. There is no known therapy for this chronic liver disease. Corticosteroids do not seem to be of benefit. This study is aimed at identifying a viral antigen or marker that might be used to screen blood for the presence of this virus. This study is also designed to characterize the natural history of this disease, so that persons who need to be treated for their liver disease can be readily identified.

Proposed Course:

We plan to continue to follow the thirteen patients already admitted to this study and to admit further patients with chronic non-A, non-B hepatitis as they are identified in the post-transfusion hepatitis studies conducted at the NIH. The major areas of proposed study will be immunofluorescence and electron microscopy of liver biopsies with particular emphasis on attempts to identify and define changes specific to non-A, non-B post-transfusion hepatitis.

Publications:

1. Hoofnagle, J.H.: Current concepts in viral hepatitis. Arquivos de Gastroenterologica Sao Paulo 16: 124-132, 1979.

2. Berman, M.D., Alter, H.J., Ishak, K.G., Purcell, R.H., Jones, E.A.: The chronic sequelae of non-A, non-B hepatitis. Ann. Intern. Med. 91: 1-6, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,511-01 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Controlled Trial of Chlorambucil Therapy in Primary Biliary Cirrhosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
Other:	J. H. Hoofnagle	Senior Investigator	DDB:NIAMDD
	D. F. Schafer	Clinical Associate	DDB:NIAMDD
	S. P. James	Clinical Associate	DDB:NIAMDD
	G. M. Dusheiko	Clinical Associate	DDB:NIAMDD
	G. Y. Minuk	Clinical Associate	DDB:NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1 1/2	0

CHECK APPROPRIATE BOX(ES)

[X] (a) HUMAN SUBJECTS [X] (b) HUMAN TISSUES [ ] (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Primary biliary cirrhosis (PBC) is a uniformly fatal disease characterized by slowly progressive intrahepatic cholestasis due to a chronic non-suppurative destructive cholangitis involving the septal and larger interlobular bile ducts. The disease is associated with multiple defects of immunologic function, hepatic copper overload and a high incidence of various autoimmune diseases. Trials of corticosteroids, azathioprine and d-penicillamine have not shown any of these agents effectively inhibit the progression of the disease or increase survival. Because certain chronic inflammatory diseases appear to respond favorably to alkylating agents but not to azathioprine, a controlled randomized trial of an alkylating agent for the treatment of PBC has been initiated. The alkylating agent chosen for the trial was chlorambucil. So far 14 patients have been admitted to the trial. Each patient undergoes a comprehensive medical evaluation prior to randomization and again after being in the trial for one and two years. Each evaluation includes a needle biopsy of the liver. In those receiving the drug total white cell counts are being maintained between 1000 and 3000/mm^3 in the presence of relative lymphocytopenia.

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Project Description

Objectives:

The objective is to conduct a controlled trial of therapy with the alkylating agent chlorambucil in patients with primary biliary cirrhosis (PBC) to determine if therapy with this agent decreases the rate of progression of the disease process or increases survival.

Methods Employed:

1) Approximately 25 patients with clinical, serum biochemical, serologic and hepatic histologic features consistent with the diagnosis of PBC are being selected for the trial. Patients must have symptoms attributable to PBC. Reasons for excluding patients include: over 65 years old, a serum biliirubin greater than 10 mg/ml and clinical features of decompensated chronic hepatocellular disease.

2) Patients undergo an initial comprehensive medical evaluation, which includes a needle biopsy of the liver. They are then randomized into a group which receives chlorambucil and a group which receives no chlorambucil, by opening sealed envelopes. Patients in both groups receive all of the conventional supportive treatments for the various manifestations of PBC as indicated. The dose of chlorambucil is adjusted so that the total white cell count is maintained between 1000 and 3000/mm^3.

3) All patients are subjected to further comprehensive medical evaluations, which include repeat needle biopsies of the liver, after being in the trial for one year and two years.

Major Findings:

In patients admitted to the trial who are receiving chlorambucil, total white cell counts are being maintained between 1000 and 3000/mm^3 in the presence of relative lymphocytopenia. It is too early in the course of the trial to determine whether chlorambucil therapy has any beneficial effects on the course of patients with PBC.

Significance to Biomedical Research:

Primary biliary cirrhosis (PBC) is a uniformly fatal disease characterized by slowly progressive intrahepatic cholestasis due to a chronic nonsuppurative destructive cholangitis involving the septal and larger interlobular bile ducts. The disease is associated with multiple defects of immunologic function, hepatic copper overload and a high incidence of various autoimmune diseases. Trials of corticosteroids, azathioprine and d-penicillamine have not shown that any of these agents effectively inhibit the progression of the disease or increase survival. Because certain chronic inflammatory diseases appear to respond favorably to alkylating agents but not to azathioprine, a controlled randomized trial of an alkylating agent for the treatment of PBC has been initiated. The alkylating agent chosen for

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IMPORTANT: (1) SINGLE SPACE, EXCEPT BETWEEN PARAGRAPHS.
(2) USE REASONABLY NEW BLACK TYPEWRITER RIBBON, PREFERABLY CARBON PAPER RIBBON.
(3) "CARBON REPRODUCTION" RIBBON IS NOT NECESSARY OR DESIRABLE.

TYPING GUIDE SHEET

START

the trial was chlorambucil rather than cyclophosphamide because of the less severe toxicity associated with the use of the former drug.

Proposed Course:

It is anticipated that the trial will take a period of approximately 3 years to complete.

Publications: None

IMPORTANT: (1) SINGLE SPACE, EXCEPT BETWEEN PARAGRAPHS.
(2) USE REASONABLY NEW BLACK NON-CARBON RIBBON, PREFERABLY CARBON PAPER RIBBON.
(3) "OFFSET REPRODUCTION" RIBBON IS NOT NECESSARY OR DESIRABLE.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,512-01 DDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of the Uptake of Lipoproteins by Hepatic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Liver Diseases Section	DDB:NIAMDD
Other:	J. A. Summerfield	Guest Worker	DDB:NIAMDD
	J. Vergalla	Chemist	DDB:NIAMDD
	E. Schaefer	Senior Investigator	MD:IR:NHLBI
	B. Brewer	Chief, Mol. Dis. Br.	MD:IR:NHLBI

COOPERATING UNITS (if any)
Molecular Diseases Branch, NHLBI

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIAMDD, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	1/2	1/4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are being designed to determine which of the major classes of hepatic cells (parenchymal, endothelial and Kupffer) are responsible for the uptake of different lipoproteins by the liver and to define which apoproteins mediate the uptake of lipoprotins by the different cell types. To faciliate the generation of suspensions of hepatic cells for these studies rat livers are perfused in situ with a solution of collagenase. The basic technique has been modified by including in the perfusate soybean trypsin inhibitor to reduce tryptic damage to cell surface receptors and deoxyribonuclease to inhibit DNA-mediated aggregation of cells. A method has been developed to separate gently endothelial and Kupffer cells from erythrocytes and parenchymal cells which involves Percoll gradient centrifugation. Endothelial cells are separated from Kupffer cells by centrifugal elutriation. The degree of separation achieved appears to be satisfactory as judged by selective stains for Kupffer cells (e.g. tartrate resistant acid phosphatase and endogenous peroxidase). The viability of cell preparations is being assessed by measuring cell membrane potentials. The first ligand to be used in these studies is radiolabeled human low density lipoprotein.

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Z01 AM 53,512-01 DDB

Project Description:

Objectives:

To determine which of the major classes of hepatic cells (parenchymal, endothelial, Kupffer) are responsible for the uptake of different lipoproteins by the liver and to define which apoprotiens mediate the uptake of lipoproteins by the different cell types.

Methods Employed:

1. To facilitate the generation of suspensions of hepatic cells for these studies rat livers are perfused in situ with a solution of collagenase. The basic technique has been modified by including in the perfusate soybean trypsin inhibitor to reduce tryptic damage to cell surface receptors and deoxyribonuclease to inhibit DNA-mediated aggregation of cells.

2. Endothelial and Kupffer cells are separated gently from erythrocytes and parenchymal cells by Percoll gradient centrifugation.

3. Endothelial cells are separated from Kupffer cells by centrifugal elutriation.

4. The viability of cell preparations is assessed by measuring cell membrane potentials.

5. Lipoproteins are isolated from rat and human serum by preparative ultracentrifugation.

6. Apoproteins are isolated using column chromatography

7. Isolated lipoproteins and apoproteins are labeled with radioiodine using the iodine monochloride or chloramine T technique.

Major Findings:

Preparations of hepatocytes, endothelial cells and Kupffer cells have been isolated from rat liver. The degree of separation of endothelial cells from Kupffer cells appears to be highly satisfactory as judged by selective histochemical stains for Kupffer cells (e.g. tartrate resistant acid phosphatase and endogenous peroxidase).

Significance to Biomedical Research:

Abnormal levels of low and high density lipoproteins in the blood have been implicated in the pathogenesis of atherosclerosis. Factors responsible for regulating levels of lipoproteins in the blood include synthesis by the intestine and liver and catabolism by the liver and peripheral tissues. This study is designed to examine the mechanisms involved in the catabolism of lipoproteins by the liver.

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Proposed Course:

The uptake of a variety of human and rat radioiodinated lipoproteins and apoproteins by different cell types of the rat liver will be studied.

Publications: None.

IMPORTANT: (1) SINGLE SPACE, EXCEPT BETWEEN PARAGRAPHS.
(2) USE REASONABLY NEW BLACK TYPEWRITER RIBBON, PREFERABLY CARBON PAPER RIBBON.
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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54016-05 DD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Lipoprotein composition in Indians and Caucasians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.V. Howard	Assistant Chief	PCRS, NIAMDD
Other:	S.L. Aronoff	Chief	PCRS, NIAMDD
	J. Reitman	Staff Physician	PCRS, NIAMDD
	P.H. Bennett	Chief	EFSB, NIAMDD

COOPERATING UNITS (if any) E. Nikkila, M. Taskinen, Univ. of Helsinki, Finland
R.H. Unger, M.D., U. of Texas, Southwestern Medical School, Dallas, TX
J. Fuller, M.D. and M. Mattock, Ph.D., Guys Hospital, London, England
D. Steinberg, M.D., U. of CA, San Diego

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.3	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Recent studies have implicated the importance of high density lipoprotein cholesterol as being an independent risk factor in the genesis of atherosclerosis. Studies have shown that individuals with a low amount of high density lipoprotein cholesterol tend to be at more risk for the development of ischemic heart disease and myocardial infarction. We are studying lipoprotein composition in Indians and Caucasians. Cholesterol and triglycerides are being measured in high density lipoproteins as well as other lipoprotein subclasses isolated by ultracentrifugation. These parameters are being compared in groups of normal and diabetic Indians with and without coronary artery disease, and the influence of therapy is being assessed. Apoprotein composition of specific lipoproteins will be measured. In addition, free fatty acid levels are being studied because of their precursor relationship to lipoproteins and their reflection of insulin action. From this study we will attempt to determine if diabetes is associated with changes in serum lipoproteins, and to delineate the importance of high density lipoprotein cholesterol values in the pathogenesis of ischemic heart disease.

Project Description:

Objectives:

1. Determination of Lipoprotein composition in normal and diabetic Indians. Previous studies have yielded data on total serum cholesterol and triglyceride levels in the Pima Indian population. In this study lipoprotein classes will be isolated by ultracentrifugation and cholesterol and triglyceride quantified in the isolated lipoproteins. These parameters will be compared in normal and diabetic individuals, and in diabetics before and after therapy to determine if diabetes is associated with changes in lipoprotein composition and how these changes are related to therapy and complications.

2. Studies of the relationship between HDL cholesterol and ischemic heart disease. Recent studies have suggested that there may be a relationship between HDL cholesterol levels and atherosclerotic heart disease. Our objective is to quantify HDL levels and study HDL composition in a series of normal and diabetic Indians with and without coronary artery disease. Study of HDL in this homogeneous and well characterized population should clarify the role of this lipoprotein in the etiology of atherosclerosis.

Major Findings:

1. Determination of lipoprotein composition in normal and diabetic Indians.

a. Influence of diet therapy on lipoproteins. Plasma lipoprotein cholesterol and triglyceride levels were measured in 24 obese Pima Indian diabetics before and after 1-8 months on a 500 calorie diet. After the period of weight loss total plasma cholesterol had declined greater than 20% and LDL cholesterol decreased 25%. The ratio of HDL to LDL cholesterol was significantly increased in all diabetics studied. Plasma total and VLDL triglyceride levels decreased in those with initial triglycerides greater than 150 mg/dl. The increases in HDL to LDL cholesterol in the diabetics correlated significantly with the changes in 2 hr. post-load glucose levels suggesting that the change in lipoprotein cholesterol distribution was induced by improved glucose tolerance. The results indicate that weight loss in maturity onset diabetics not only improves glucose tolerance but also reverses the plasma lipid changes associated with this disorder.

b. Free fatty acid levels during glucose tolerance testing in diabetics. Changes in free fatty acid and insulin levels during OGTT and IVGTT have been determined in 12 obese Pima Indians with severe diabetes, six obese Pimas with mild diabetes, 11 obese non-diabetic Pimas, and 10 obese non-diabetic Caucasians. During OGTT, the decline in free fatty acid levels in the severe diabetics were of the same magnitude as those of the Pima and Caucasian non-diabetics (278±55 vs. 284±32 and 262±43 Eq/L). This occurred despite an insulin increment in the diabetics of only 7±2 μU/ml as compared to 252±34 and 75±18 for the Pima and Caucasian controls. IVGTT in the severe diabetics produced no insulin response and free fatty acids

did not decline. In the less severe diabetics increments in insulin averaged 15±4 μU/ml during IVGTT, while free fatty decreases were 214±53 μEq/L. This was again similar to Pima and Caucasian controls whose free fatty acid changes were 234±37 and 183±51 μEq/L with insulin responses of 216±23 and 124±56 μU/ml, respectively. Assay of plasma glycerol showed declines parallel to those of free fatty acids, indicating that the free fatty acid changes in the diabetic were attributable to inhibition of lipolysis rather than to increased removal. These findings demonstrate marked sensitivity to the antilipolytic effects of insulin in individuals considered to be resistant to its glucose-lowering action.

c. Relation between fatty acid levels and somatostatin secretion. To investigate the effect of acute elevation of plasma free fatty acids on the secretion of splanchnic somatostatin-like immunoreactivity (in collaboration with Dr. Unger) the peripheral venous pancreatic and gastric venous effluent levels of SLI were measured in normal and chronic alloxan diabetic dogs before and after the infusion of a fat emulsion supplemented with heparin. In normal conscious dogs heparin, injected during the infusion of a fat emulsion, elevated FFA levels from a mean (± SE) baseline level of 0.7±0.1 mEq/l to a peak value of 1.5±0.1 mEq/l ($p<0.001$) and plasma SLI rose from a mean (± SE) baseline value of 145±7 pg/ml to a peak of 253±44 pg/ml ($p<0.05$). Neither the infusion of glycerol, of fat emulsion without heparin, of heparin alone nor of saline itself had an effect on either the plasma level of FFA or SLI. In another group of anesthetized dogs with surgically implanted catheters the administration of fat emulsion plus heparin was accompanied by more than a two-fold rise in the concentration of SLI in the venous effluent of the pancreas and of the gastric fundus and antrum in association with an elevation of FFA levels. In a group of conscious diabetic dogs fat emulsion plus heparin raised FFA from a mean baseline level of 1.2±0.2 mEq/l to 1.6±0.3 mEq/l ($p<0.05$) and SLI rose from a mean baseline level of 185±9 pg/ml to a peak value of 310±44 pg/ml ($p<0.01$). Although SLI levels were significantly greater than in normal dogs at several time points after the rise in FFA, the magnitude of the increment in diabetic dogs did not differ from normal. These results demonstrate that a rise in FFA levels is a potent stimulus for SLI secretion from the pancreas and stomach and raise the possibility that FFA is an important physiological regulator of SLI secretion.

d. Cross sectional study of lipoprotein composition and HDL subfractions in the Pima Indians. Blood sampling to measure lipoprotein triglycerides and cholesterol has continued on all individuals over age 15 seen for biennial exams at SFSS. In addition, HDL subfractions are being isolated from all individuals between 35-44 years. Cholesterol and triglycerides have been measured in all lipoprotein fractions and they have been stored frozen for future apoprotein studies. We have participated in a standardization program with the CDC, Atlanta, Georgia, in order to insure that our plasma lipid measurements will be comparable to those of the Lipid Research Clinics. In collaboration with Drs. Fuller and Mattock in London and Drs. Nikkila and Taskinen in Helsinki comparable groups of Caucasian diabetics have been recruited and plasma lipoproteins are being analyzed.

To insure comparability of methods, plasma samples and cholesterol and triglyceride controls have been exchanged at approximately six month intervals. To date approximately 1000 Pima samples have been quantified.

e. Studies of the effect of oral contraceptives on bile and lipoprotein composition. Oral contraceptive usage results in an increase in the degree of cholesterol saturation of human gallbladder bile. To characterize the mechanisms responsible for this effect we measured biliary lipid secretion, gallbladder and hepatic bile lipid composition, bile acid composition and pool size, and plasma lipoprotein levels in five healthy women during routine usage of oral contraceptives, and again in the same five women after resumption of normal menstrual function following the cessation of oral contraceptive usage. We found that stimulated hepatic bile, as well as gallbladder bile, was more highly saturated with cholesterol during oral contraceptive usage than afterwards ($p<.02$), suggesting that the lithogenic effect of oral contraceptives was a significant enhancement of biliary cholesterol secretion ($p<.01$), observed in every subject. Contrary to expectations based on animal studies, oral contraceptive usage did not interfere with bile acid secretion except in one subject. Phospholipid secretion was actually slightly, although not significantly, higher during oral contraceptive usage than during normal menstrual function. Bile acid pool size did not change significantly following discontinuation of oral contraceptive usage and resumption of normal menstrual function. Bile acid composition, however, showed a diminution of the percent chenodeoxycholic acid during oral contraceptive usage. This observation suggests that chenodeoxycholic acid pool size, smaller in each patient during oral contraceptive usage, may be an important determinant of the hypersecretion of cholesterol observed during oral contraceptive therapy. Total and very low density lipoprotein triglyceride levels and total and low density lipoprotein cholesterol levels were higher during oral contraceptive treatment than during normal menstrual cycles, but did not correlate with the secretion rates measured for biliary cholesterol, bile acid or phospholipid. Plasma high density lipoprotein cholesterol levels did not change in any consistent or significant manner with oral contraceptive usage, and there was no significant correlation between HDL cholesterol levels and biliary lipid secretion rates. We conclude that oral contraceptives raise the cholesterol saturation of human gallbladder bile by increasing the hepatic secretion of cholesterol into bile, and that this effect may be related to alterations in chenodeoxycholic acid levels.

2. Studies on the relationship between HDL cholesterol and ischemic heart disease.

a. Lipoprotein composition has been determined in six diabetic Indians who have had documented myocardial infarcts, and in six controls matched for tribe, age, sex, weight, and diabetic status. Data in this small group showed no significant differences in plasma lipoproteins in the individuals with MI.

b. Individuals in the Pima population with evidence of EKG abnormalities have been identified and plasma samples are being obtained at the Sacaton clinic from these individuals at the time of their biennial examination. Lipoprotein cholesterol and triglycerides are being measured and HDL subfractions isolated from these and from controls matched for age, sex, weight and diabetic status. Comparable groups of Caucasians with EKG abnormalities have been identified by Drs. Fuller and Mattock in London and Drs. Nikkila and Taskinen in Helsinki for similar lipoprotein quantification. To date 50 Pimas with EKG changes have been identified and analyzed.

Significance to Biomedical Research and the Program of the Institute: This study will determine whether changes in lipoprotein composition, and especially HDL cholesterol levels, are associated with diabetes and the development of ischemic heart disease. The data obtained on HDL subclasses will help elucidate the relationship of this lipoprotein to the development of diabetes and atherosclerosis. Since this population serves as a carefully defined model for adult onset diabetes, the data should contribute to an understanding of the effects of diabetes on lipoprotein metabolism and the relationship of lipoproteins to the development of both atherosclerosis and microvascular complications.

Proposed Course:

1. Determination of lipoprotein composition in normal and diabetic Indians.

a. Comparison of non-diabetics and diabetics. Assays of lipoprotein cholesterol and triglycerides in all individuals over age 15 seen for biennial exams will be continued in order to obtain enough samples for analyses by age groups within the normal and diabetic population. Data analysis will begin and results of Pima lipoprotein patterns will be compared to Caucasian diabetics selected and assayed by Drs. Fuller and Nikkila.

b. Studies on HDL. Measurements will continue of the HDL composition in 35-44 year old diabetic and non-diabetic Pima Indians. In addition, a radioimmunoassay will be developed with Dr. Fuller to measure apo A-1 and apo A-2 levels in the HDL fractions and to compare these in HDL from non-diabetics and diabetics. Analysis of the data on individuals with EKG changes will begin and results will be compared to the Caucasian data.

c. Assay of plasma samples from individuals who died of arteriosclerotic heart disease. Individuals with ASHD documented at autopsy will be identified and compared to controls autopsied for other purposes. Serum samples from previous biennial exams are available and will thawed for measurement of total and HDL cholesterol.

Publications:

Howard, B.V., Savage, P.J., Nagulesparan, M., Bennion, L.J., Davis, M., and Bennett, P.H.: Changes in plasma lipoproteins accompanying diet therapy in obese diabetics. Atherosclerosis 33:445-56, 1979.

Howard, B.V., Savage, P.J., Nagulesparan, M., Bennion, L.J., Unger, R.H., and Bennett, P.H.: Evidence for marked sensitivity to the antilipolytic action of insulin in obese maturity-onset diabetics. Metabolism 28:744-50, 1979.

Wasada, T., Howard, B., Dobbs, R.E., and Unger, R.H.: Evidence for a role of FFA in the regulation of somatostatin secretion in normal and alloxan diabetic dogs. J. Clin. Invest., in press 1980.

Bennion, L.J., Mott, D.M., and Howard, B.V.: Oral contraceptives raise the cholesterol saturation of bile by increasing biliary cholesterol secretion. Metabolism 29:18-22, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54017-04 DD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effects of Weight-reduction on glucose tolerance and gluco-regulatory hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.S. Rietman	Staff Physician	PCRS, NIAMDD
Other:	S.L. Aronoff	Chief	PCRS, NIAMDD
	M. Nagulesparan	Guest Worker	PCRS, NIAMDD

COOPERATING UNITS (if any)
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LAB/BRANCH
Digestive Disease Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	1.2	2.0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of weight loss in obese diabetic and non-diabetic adults will be supplemented by studies employing exercise training in similar individuals to help elucidate the inter-relationship of glucose tolerance, insulin sensitivity and gluco-regulatory hormone levels. The effects of exercise training or weight loss on glucose tolerance tests, glucose disposal during glucose clamp, insulin secretory responses to various stimuli, 24 hr. urinary C-peptide excretion, tissue lipoprotein lipase activities, and body composition will be examined. The magnitude of the exercise training effect will be followed by measurements of maximal oxygen uptake capacity. Measurement of heat production (or thermogenesis) during weight loss or gain will suggest whether differences in energy conservation predispose toward obesity in the Pima Indians. These studies will provide much needed information on the effectiveness and limitations of weight loss and/or exercise programs in the treatment of obesity and type II diabetes.

Project Description:

Objectives: Many components of the natural history of diabetes remain to be described. A major difficulty is the absence of sequential data on individuals in transition from the normal to the diabetic state. Furthermore, there are differences in gluco-regulatory phenomena between recent-onset and long-term diabetics. The study of obese subjects with borderline glucose tolerance and those with recent-onset and long-term diabetes appears likely to reveal much about the interrelationship of glucose tolerance and gluco-regulatory hormone levels.

Major findings to date include the observation that oral glucose tolerance and insulin response improve with weight reduction in recent-onset diabetics, while i.v. glucose tolerance remains impaired with a flat insulin response.

As a logical extension of these findings, we propose to use exercise training as an additional intervention in the obese non-diabetic and recent-onset diabetic subjects. Several studies have suggested that peripheral sensitivity to the gluco-regulatory effects of insulin is improved with exercise training in both normal and obese, non-diabetic subjects. Characterizing the changes in glucose tolerance and gluco-regulatory hormones with exercise training is therefore a major objective of this protocol.

Methods Employed: Obese diabetics with onset of symptomatic glucose intolerance within the past 4 years will be studied and obese non-diabetics will be admitted as controls.

Subjects undergoing weight loss will initially be placed on a weight maintaining 45% CHO, 40% fat, and 15% protein diet and have oral and intravenous glucose tolerance tests, and test meals with measurement of glucose, insulin GIP, and glucagon responses. Subjects will then be placed on a 500 calorie (including at least 500gm carbohydrate) diet to induce rapid weight loss. Subjects will be refed with a 45% CHO, 40% fat, and 15% protein weight maintenance diet and after weight stabilization the initial series of tests will be repeated. Subjects in the exercise study will be placed on weight maintenance diets containing 45% CHO, 35% fat, and 20% protein. At certain intervals they will undergo oral and i.v. glucose tolerance tests, collection of 24 hr. urinary for C-peptide determination and measurements of insulin resistance by the glucose clamp technique. Furthermore, maximal oxygen uptake and body density determinations before and after exercise training (while strictly maintaining weight) will be measured.

Major Findings: Initial studies were conducted on 10 recent-onset diabetic and 10 non-diabetic subjects. Fasting and 2 hour plasma glucose levels of the diabetics showed highly significant improvements: 160 ± 8 mg/dl to 98 ± 4 mg/dl ($p<0.001$) and 412 ± 12 mg/dl to 203 ± 10 mg/dl ($p<0.001$), respectively. Insulin secretion in response to both oral and intravenous glucose improved significantly in the diabetics ($p<0.05$) while decreasing in the controls. Although the glucose disposal rate during the intravenous glucose tolerance test improved significantly in the diabetics, it remained

significantly below that of the controls. The acute insulin response to IV glucose increased significantly but was only 12% of that seen in the controls. In contrast, insulin responses to arginine were similar in the diabetics and controls. Glucagon suppression by intravenous glucose and stimulation by intravenous arginine improved but could not be entirely normalized. Insulin binding to circulating mononuclear cells was initially similar in the two groups and increased in both following weight loss.

In the study of patients with Type II diabetes with a duration of five years or longer, treatment with hypocaloric diet and weight reduction led to improvement in both fasting and post-challenge glucose levels in all subjects. Normal fasting plasma glucose levels were achieved in most of the short-term diabetic subjects but not so in the long-term group. In the long-term diabetic group there was a slight but not significant increase in plasma insulin response to oral glucose on the final as compared to the initial test, indicating little recovery of endogenous B-cell function. In contrast, the improvement in the short-term group was associated with a fall in fasting plasma insulin levels and an increased insulin increment above fasting levels in response to oral glucose on the final as compared to the initial test. This indicates that there was considerable recovery of the ability of the B-cell to respond to an oral glucose load in subjects with short-term diabetes, but not so in the long-term diabetic group. In the final tests, the plasma insulin responses to oral glucose were similar in both the short- and long-term diabetic groups, but the oral glucose tolerance was considerably better in the short-term than the long-term group. This finding suggests that the improvement in glucose tolerance seen in short-term diabetics was also due to an increased sensitivity of the body to insulin-mediated glucose disposal. Although the short-term diabetics lost less weight than the long-term diabetics, they still showed an improvement in B-cell function and insulin sensitivity.

Studies have also been completed on 9 subjects with borderline or impaired glucose tolerance. In contrast to both the short- and long-duration overt diabetics, insulin secretion in these subjects was initially high and decreased following dietary therapy despite significant improvements in the glucose tolerance of the subjects. These responses were similar to those observed in the obese control group discussed above.

Preliminary studies of obese non-diabetic and non-obese diabetic subjects before and after training have revealed no change in oral glucose tolerance after training in either the diabetic or the obese subjects. However, the obese has improved i.v. glucose tolerance in spite of a substantial decrease in insulin response to i.v. glucose with training. In the diabetic there was no improvement in insulin secretion with i.v. glucose and questionable improvement in i.v. glucose disposal.

The findings indicate that maturity onset diabetics have a selective defect of insulin secretory response to a glucose stimulus and that, early in the course of the diabetes this defect is substantially reversible. The insulin binding studies suggest that diminished insulin sensitivity may contribute

to pancreatic decompensation in individuals unable to compensate with hypersecretion of insulin. No evidence was found of a primary defect in receptor binding in maturity onset diabetes. The overall study to date indicates that resistance to the action of insulin may be the initial lesion as glucose tolerance begins to deteriorate and that this is followed by progressive failure of insulin secretion in response to a glucose challenge.

Significance to Biomedical Research and the Program of the Institute: These studies have a good prospect of differentiating the reversible and irreversible abnormalities that characterize diabetes mellitus. It is possible that this study may identify changes that are reversible in recent onset diabetics that become irreversible with prolonged duration of hyperglycemia. These findings can be utilized to improve our methods of treating maturity onset diabetes.

The potential use of exercise to treat diabetes has been touted as a panacea because of the well known acute effects of exercise on peripheral glucose disposal, particularly in non-insulin dependent diabetics. However, few studies have addressed the issue of whether exercise training really produces any improvement in those metabolic parameters which define the diabetic state. By attempting to define the effects of exercise on glucose tolerance, pheripheral sensitivity to insulin, B-cell secretory response to various stimuli, body composition and lipogenic enzyme activity we will clarify the extent of the long-term usefulness of exercise as a part of the treatment of diabetes.

Proposed Course: We plan to initiate a new weight loss study in parallel to the exercise study mentioned above. We will extend our observations on the changes in glucose tolerance with weight loss to include measurements of insulin, glucose and GIP levels.

The exercise studies will be extended to include 4-6 obese diabetics and several more obese non-diabetics. Several more tests will be included in the protocols:

1. Fat and muscle biopsies for tissue lipoprotein lipase activity, before and after exercise or weight loss (see Z01 AM 54 023-03).
2. Measurement of 24 hr. urinary C-peptide excretion before and after exercise or weight loss to examine the relationship of B-cell function to glucose disposal.
3. Maximal oxygen uptake determinations during exercise training to provide a measure of the magnitude of the training effect, so that any observed changes in LPL activity, gluco-regulatory phenomena or body density may be correlated with the magnitude of the training effect achieved.
4. Body density measurements to define any changes in body composition occurring during exercise.
5. Glucose clamp studies to measure changes in in vivo sensitivity to the glucose-lowering effects of insulin (not adequately defined by monocyte or adipocyte receptor studies) with exercise.

Finally, we intend to perform studies on individuals combining exercise and weight loss to determine (1) whether both interventions together improve the overall gluco-regulatory picture more than either alone and (2) whether body composition changes during extensive weight loss are favorably influenced by a simultaneous exercise program.

Publications:

Savage, P.J., Bennion, L.J., Flock, E.V., and Bennett, P.H.: Beta cell Dysfunction in maturity-onset diabetes: Reversible loss of glucose-induced insulin secretion with retention of response to arginine. In Camerini-Davalos, R.A. (ed.): Treatment of Early Diabetes. New York, Plenum Press, 1979, pp. 219-225.

Savage, P.J., Bennion, L.J., Flock, E.V., Nagulesparan, M., Mott, D.M., Roth, J., Under, R.H., and Bennett, P.H.: Diet-induced improvement of abnormalities in insulin and glucose secretion and in insulin receptor binding in diabetes mellitus. J. Clin. Endoc. Metab. 48:999-1007, 1979.

NIH Contract No. N01 AM-6-2219 Starting Date: September 30, 1976
Expiration Date: August 31, 1979

Contractor: University of Texas Health Science Center
Southwestern Medical School

Money Allocated: 360,000.00 Man Years Purchased: 13

This contract enables extension of studies carried out under the NIH Project Z01 AM 54017-04, Effect of Weight Reduction on Glucose Tolerance and Glucose Regulatory Hormones.

We are planning to initiate studies concerning changes in body energy conservation with weight gain or loss. These questions have arisen from the lack of knowledge of whether individuals with a propensity toward obesity have more efficient mechanisms for energy conservation than naturally lean individuals. The possibility that alterations in thyroid and/or catecholamine metabolism accompany weight gain and loss has been suggested by preliminary studies in Caucasian subjects at the U. of Vermont (subcontracted by U. of Texas under the above Contract). The study will deal primarily with measurements of thermogenesis (heat production) during various nutritional states in obese and lean individuals. Substantial differences in thermogenesis between lean and obese individuals in their responses to over- or undernutrition would perhaps suggest an underlying predisposition (possibly genetically determined) toward obesity. Further, the classification of obese individuals according to their thermogenesis profile would be compared with other markers for obesity such as adiposity, body density, cell size, LPL activity, etc. in an effort to determine the predictability of ease of weight loss therapy in these individuals.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54018-04 DD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Characterization of Metabolic and Morphologic Abnormalities in Diabetes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.4	1.4	2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Age, sex and weight matched groups of genetically normal, prediabetic and diabetic Pima Indians and normal Caucasians were examined by means of oral and intravenous glucose tolerance tests, arginine infusions, in vivo insulin resistance measurements, and circulatory mononuclear cell insulin receptor assays, and urinary protein profiles to determine whether abnormalities of insulin, glucagon secretion or other measurements are characteristic of the prediabetic and diabetic state.

Project Description:

This investigation is a continuation and expansion of the previous projects: 1) Evaluation of the Prediabetic and Diabetic State in the Pima Indians (Z01 AM 69,003-04 EFSB), 2) Evaluation of the Prediabetic State in the Pima Indians-Caucasian Controls (Z01 AM 69,004-04 EFSB), 3) Evaluation of Basement Membrane Thickness in Diabetic Pima Indians (Z01 AM 69,005-04 EFSB).

Objectives: Many components of the natural history of diabetes remain to be described. A prospective study of genetically suspect prediabetic subjects (defined as the non-diabetic offspring of two diabetic parents) appears to be the most direct and reliable method of describing the natural history of the disease.

Study of the prediabetic will enable us to better understand the interrelationship of glucose levels with insulin and glucagon levels, an area which has remained controversial. These studies will also clarify the relationship of hyperglycemia to microangiopathy. Detailed studies of urinary protein abnormalities and muscle capillary basement membrane thickness have been examined in the Pima and should clarify the history of some of the earliest physical abnormalities found in diabetes.

The purpose of the study are:

1. To determine whether there are abnormalities in carbohydrate metabolism gluco-regulatory hormone secretion, urinary protein excretion or capillary basement membrane thickening in non-diabetic Pima Indians compared with non-diabetic Caucasian controls.

2. To determine if there are differences in these variables bewteen Pima Indians with 2 parents with diabetes and those with 2 non-diabetic parents.

3. To initiate a long term prospective study of prediabetes.

Methods Employed: Four groups were selected for study - Pima Indian non-diabetic offspring of two diabetic parents, non-diabetic offspring of two non-diabetic parents, subjects with long and short duration of documented diabetes and non-diabetic Caucasians with two non-diabetic parents. The non-diabetic groups were matched for age, sex and percent desirable weight.

Following dietary preparation all subjects were admitted to the Clinical Research Center for testing. Tests included an oral glucose tolerance test, intravenous glucose tolerance test, cortisone glucose tolerance test, an arginine infusion, a quadriceps needle muscle biopsy, fundus photographs and 24-hour urine collected for determination of specific urinary proteins by nephelometry.

Two sub-groups of diabetics were examined a) long-term diabetics, defined as subjects with fasting plasma glucose $\geq$ 140 mg/100 ml or a two hour post-

carbohydrate load glucose >300 mg/100 ml and >10 years duration with and without retinopathy, and b) short-term diabetics defined as subjects who have had a documented marked deterioration in their oral glucose tolerance over the last 5 years from a two hour post-carbohydrate load plasma glucose $\leq$140 mg/100 ml to a two hour plasma glucose $\geq$ 300 mg/100ml.

The very high prevalence of diabetes in the Pima people, while providing a very large group of genetically prediabetic individuals, raised a problem of finding individuals who are truly normal. Genetically normal Pima offspring were selected from phenotypically normal parents 45 years of age or older. It is conceivably possible, however, that with the high prevalence of diabetes in the tribe, virtually all the Pimas could carry the diabetic genome.

Because of this possibility, a group of non-diabetic Caucasians with non-diabetic family histories were examined as an additional control group to compare with the "normal" Pimas.

Results:

1. Insulin levels in the Pima. The pattern of insulin response to oral and intravenous glucose has been claimed to be characteristic of diabetes and prediabetes. While insulin responses to oral glucose were reduced in subjects with 2 hour glucose levels over 225 mg/dl and the acute phase insulin release was absent in all diabetics there was evidence of hyperinsulinemia in the Pima non-diabetics compared to the Caucasians. The normal and prediabetic Indians had fasting and stimulated insulin levels during all the tests 2-3 times greater than those on the Caucasians. Differences in insulin levels between the two races could not be explained by differences in glucose level, age or obesity. These studies raised the possibilities of either 1) extra pancreatic insulin resistance or 2) an abnormality of biological effectiveness of Pima insulin, or 3) abnormal hepatic insulin extraction.

2. C-Peptide Studies. To assess whether hepatic insulin extraction is diminished in the Pima, serum insulin and C-peptide concentrations were determined during oral glucose tolerance tests performed on ten Pima Indians and ten Caucasians with normal glucose tolerance and a range of fasting insulin levels. The results indicate that there is no abnormality of hepatic insulin extraction in the Pimas. In addition, this study suggests that the fractional hepatic extraction of insulin does not change over a wide range of obesity and insulin levels.

3. Studies of Insulin Resistance. An improved method has been developed to measure in vivo resistance to insulin mediated glucose disposal in order to investigate the mechanism of the hyperinsulinemia and diabetes prevalent in the Pima Indians. Previously a mixture of glucose and insulin infused over 150 minutes had been used in other centers to determine in vivo insulin resistance; this method assumed that the exogenous insulin infused could suppress endogenous insulin release. In order to evaluate this assumption, a mixture of glucose (6 mg/kg/min) and insulin 80 mU/min was infused for 150 minutes in our subjects and plasma C-peptide concentrations were measured

during the infusion. Despite the presence of exogenous insulin, plasma C-peptide levels continued to increase with the increase in plasma glucose levels, indicating that endogenous insulin was being released. This study was repeated using double the amount of insulin (160 mU/min vs 80), but with the maintenance of plasma glucose levels similar to the first infusion. Despite having almost double the plasma insulin levels the plasma C-peptide concentration continued to increase, confirming that exogenous insulin over a wide concentration range could not completely inhibit the release of endogenous insulin. To completely block endogenous insulin release, we therefore infused a mixture of glucose (420 mg/min) insulin (0.77 mμ/kg/min) and somatostatin (500 μg/hr) for 150 minutes. A steady state level of plasma insulin and glucose was attained after 90 minutes. Endogenous insulin secretion determined by C-peptide measurement and glucagon remained suppressed throughout the infusion. The method is simple, safe and independent of endogenous insulin secretion.

We next employed the method to quantify in vivo resistance to insulin mediated glucose disposal in group of subjects with a wide range of obesity and normal glucose tolerance. With similar steady state levels of plasma insulin (SSPI) maintained in all subjects the height of the steady state plasma glucose (SSPG) concentration was considered an index of total body sensitivity. A positive correlation between SSPG and the degree of obesity [Body Mass Index (BMI)] was demonstrated. The fasting plasma insulin concentration correlated with BMI and SSPG. Therefore, by adjusting insulin according to body weight and keeping the glucose infusion constant, this method can be used to study subjects over a range of obesity.

The above method was next used to determine whether insulin resistance of obese glucose intolerant subjects can be accounted for by obesity alone. Fourteen obese glucose intolerant and twenty-one diabetic Southwestern American Indians with similar degree of obesity were studied. The mean SSPG was higher in the glucose intolerant subjects than in weight matched subjects with normal glucose tolerance (226 ± 10 vs 136 ± 13 mg/dl $p<0.0001$) indicating that obesity was not the only factor responsible for the increased resistance in obese glucose intolerant subjects. This increased resistance to insulin action was found in the presence of similar insulin binding to mononuclear cells. It appears that in obese Southwestern American Indians with glucose intolerance, abnormalities beyond the site of insulin binding to the receptor may explain the observed increase in in vivo insulin resistance.

We have also sought to determine whether the Pima Indians with normal glucose tolerance have increased in vivo insulin resistance compared to weight matched Caucasian subjects. The findings so far using the somatostatin method indicate that there is no difference in in vivo insulin resistance between these two groups. For this study we have also employed the euglycemic clamp technique of Andres et al. Under euglycemic conditions there was also no difference in insulin mediated glucose disposal between the two groups.

Other studies are in progress to determine insulin sensitivity in genetic prediabetic Pima Indian subjects and to investigate changes in insulin resis-

tance after control of diabetes or dietary manipulations.

4. Urinary Protein Excretion in Diabetics. This study was designed to determine if good control of plasma glucose levels in obese Type II diabetic subjects could result in a reduction in urinary protein excretion. Good control of plasma glucose levels were obtained by a period of total fast for 7 days followed by a low calorie diet. Comparisons were made with obese subjects with normal and impaired glucose tolerance. Before the fast, the urinary protein output was higher in most of the diabetic subject, but there was no correlation between the plasma glucose level and proteinuria. In 17 of the 20 subjects studied, the total protein excretion fell on the first day, but increased during the next six days, to return to prefast levels on refeeding. The increase was relatively small in subjects with normal glucose tolerance. Most of the subjects with impaired glucose tolerance were females, who showed a distinct increase in total protein excretion. (It is not clear whether the observed increase in proteinuria in this group was dependent on the degree of glucose tolerance or on the sex of the subject. In the subjects with diabetes and elevated prefast plasma glucose levels, increase urinary protein excretion occurred despite good control of plasma glucose level during the fast. The changes in protein excretion appear to be relatively independent of the plasma glucose levels, but more dependent on the nutritional status of the patient. In many of our subjects, increases in albumin during the fast accounted for very little of the increase in total protein, and in none of the subjects did albumin account for all the total protein increase.

5. Studies of Juvenile Diabetics. Juvenile diabetes is common in Pima Indian teen-agers and ranges from mild glucose intolerance to insulin requiring diabetes. To compare Caucasian and Pima juvenile onset diabetics, 10 subjects of each race with onset of diabetes below the age of 20 years who were treated with insulin were given oral glucose tolerance tests and IV arginine infusions with measurement of plasma glucose and C-peptide concentrations. Urine was collected over 24 hour period for measurement of C-peptide concentration as an index of 24 hour endogenous insulin secretion. Pima subjects had significantly higher C-peptide levels fasting, in response to both glucose and arginine and in 24 hour urine collections. This study suggests that even the cases of severe junvenile onset diabetes found in the Pima are a different disease from that found in the majority of young Caucasians. It also indicates that C-peptide determinations may be useful in studies of the etiology of diabetes by making it easy to distinguish classic insulin-dependent diabetics within groups of insulin treated diabetics.

Significance to Biomedical Research and the Program of the Institute: These studies are providing information on issues which have been controversial in diabetes research for over a decade. They are making it possible to systematically approach the cause of diabetes in the Pima, a group with the highest known prevalence of maturity onset diabetes.

Proposed Course: Subjects will be recalled every two years for an additional series of tests. The second recall is now in progress. This should

enable careful documentation of the natural history of diabetes. Additional studies will be conducted to evaluate insulin resistance in hyperinsulinemic Pimas and to evaluate the biological activity of Pima insulin. Other methods and technique for evaluating these prediabetic and normal subjects will be incorporated in the protocol as they are proven useful.

Publications:

Nagulesparan, M., Savage, P.J., Unger, R.H., and Bennett, P.H.: A Simplified Method Using Somatostatin for the Assessment of In Vivo Insulin Resistance Over a Range of Obesity. Diabetes 28: 980-983, 1979.

Nagulesparan, M., Savage, P.J., Mott, D.M., Johnson, G.C., Unger, R.H., and Bennett, P.H.: Increased In Vivo Resistance in Obese, Glucose Intolerant Southwestern American Indians. Evidence for a Defect Not Explained by Obesity. J. Clin. Endocrin. Metab. In press, 1980.

NIH Contract No. NO1-AM-6-2219 Starting Date: September 1, 1979
Expiration Date: August 31, 1980

Contractor: University of Texas Health Science Center
Southwestern Medical School

Money Allocated: $1,699,947 Man-years Purchased: 23.7

This contract is for extension of the studies carried out under the NIH Project Z01 AM 54018-03, Characterization of Metabolic and Morphologic Abnormalities in Diabetes.

Work to date has been comprised of consultations on studies of diabetes and lipid metabolism and the measurement of glucagon, insulin, lipids and lipoproteins on samples obtained by NIH personnel and one contract physician at the Phoenix Clinical Research Center.

Studies underway utilizing contract personnel working both in Phoenix and in Dallas including further investigations of the relation of glucagon and insulin levels, attempts to restore glucagon secretion to normal by aggessive therapy of diabetes and the use of somatostatin and its analogues to investigate effects of glucagon suppression and determine extrapancreatic insulin resistance. Studies on lipid metabolism in diabetes and studies of lipoprotein turnover and interrelationships have begun. Investigation of gut hormone response to meals in Pima and Caucasian subjects are in progress.

Proposed Course: The above studies will be continued. In addition, further morphological studies of diabetic pathology utilizing electron microscopy are planned.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54019-04 DD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cellular Metabolism in Diabetic and Non-diabetic Subjects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.V. Howard	Assistant Chief	PCRS, NIAMDD
Other:	D.M. Mott	Research Biochemist	PCRS, NIAMDD
	H. Hidaka	Visiting Associate	PCRS, NIAMDD
	F. Ishibashi	Visiting Scientist	PCRS, NIAMDD

COOPERATING UNITS (if any)
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Human Diabetes Study Center, U. of TX Southwestern Medical School, Dallas
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LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	2.2	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Elevated insulin levels in Pima Indian adults compared to weight matched Caucasian controls suggest that cellular changes external to the pancreas may be important in the pathogenesis of diabetes mellitus. The aim of this project is to study several aspects of insulin function in cells obtained from normal and diabetic Pima Indians, in order to determine if diabetes mellitus is expressed in genetic differences at the cellular level. Fibroblast cultures have been established from normal and diabetic Pima Indians. Parameters of cell growth are being compared in the normal and diabetic cells, and insulin binding is being studied. In addition, cellular metabolism and transport functions are being studied in the presence and absence of insulin, in order to further investigate cell insulin action and to determine if cellular abnormalities in insulin response occur in individuals with diabetes.

PHS-6040

Project Description:

Objectives: Resistance to insulin mediated glucose uptake is a well documented component of the obesity and diabetes which are so prevalent in the Pima population. Diploid fibroblast cultures established from skin biopsies afford an easily accessible system for the examination of cellular metabolic capacity which is isolated from physiological variables. This project has dual objectives: (1) to examine the action of insulin on glucose metabolism in the fibroblast (several aspects of glucose metabolism including hexose transport, glycogen synthesis, and CO_2 production will be examined and the mechanism of insulin action on these parameters will be investigated), (2) to establish cultures from biopsies of genetic normal, prediabetic and diabetic Pima Indians and age-matched Caucasian controls and to compare sensitivity and magnitude of response to insulin in the systems developed in (1).

Major Findings:

(1) Investigations of insulin action in the cultured fibroblast.

(a) Insulin binding. The fibroblast has been demonstrated to possess a specific insulin receptor which is regulated by physiological levels of insulin. Studies of a whole cell receptor binding and results of assays in partially purified insulin receptor preparations from the fibroblast are summarized in Z01 AM 54020-02 DD.

(b) Insulin stimulation of total cell glucose incorporation. Influence of insulin on total cell glucose incorporation was evaluated using ^{14}C-glucose. Glucose incorporation was increased up to two-fold in the presence of insulin. Insulin action occurred within 30 minutes and could be observed with insulin concentrations as low as 10^{-10}M (10 μU/ml); half maximum stimulation was achieved at 3×10^{-9}M. The action of insulin was enhanced by preincubation in glucose-free medium. After glucose starvation the cells converted glucose primarily to glycogen, nucleotides, and lactate and the stimulation by insulin was observed equally in all fractions. Thus insulin stimulates several aspects of glucose metabolism in diploid fibroblast monolayers.

(c) Insulin stimulation of Hexose Transport. Influence of insulin on the kinetics of hexose transport was studied using 2-deoxyglucose and 3-O-methyl glucose. A large diffusion component was corrected using p-chloromercuribenzoic acid and phloridzin. K_m for facilitated diffusion averaged 1.9 mM for 2-deoxyglucose and 5.3 mM for 3-O-methyl glucose, and V_{max} ranged from 10-24 n moles/min/mg cell protein. Insulin resulted in a 150 percent increase in V_{max} with no significant change in K_m. Glucose starvation increased the V_{max} of hexose transport, but insulin action on transport was greater after preincubation with elevated glucose levels (16 mM).

(d) Insulin stimulation of glycogen synthase. The effect of insulin on glycogen synthase activity in human diploid fibroblasts has been studied.

As little as 2 x 10^{-10}M insulin increased the glycogen synthase I activity without changing the total activity. Stimulation occurred within 5 minutes and became maximal in 30 minutes. A half maximal increase of I activity was achieved at 3 x 10^{-9}M insulin. Glucose starvation increased the magnitude of response of glycogen synthase to insulin but did not change the insulin concentration necessary to give a half maximal stimulation. Glucose increased the basal level of I activity in human diploid fibroblasts; the effect of insulin was additive. During vitro senescence the total glycogen synthase activity declined, but the concentration of insulin which produced a half maximal stimulation remained unchanged.

(e) Influence of pH on Insulin Action. In order to elucidate the mechanism of decreased insulin action observed during diabetic ketoacidosis, the influence of pH and 3-hydroxybutyrate (3OHBA) on insulin binding and action has been studied in cultured human diploid fibroblasts. When the pH of the incubation medium was decreased from 7.6 to 6.8, insulin stimulation of glycogen synthase and total glucose uptake were decreased. The decreased pH induced both an increase in the insulin concentration for half maximal response, and a decrease in maximal responsiveness. When insulin binding was measured at lower pH, receptor affinity was decreased. The effect of 3OHBA on insulin binding and action was assessed at pH 7.4 and 6.9. In the presence of 30 HBA, insulin binding increased but insulin stimulation of glycogen synthase was unaffected. The data show that insulin action is impaired at lower pH. The decreased sensitivity is probably related to the decrease in insulin binding affinity at lower pH, but decreased maximal responsiveness implies that cellular post-receptor components are also affected by lower pH. The results indicate that in diabetic ketoacidosis the decreased pH rather than 30 HBA is the primary mediator on interference in insulin action.

(f) Action of Alloxan on Glucose Metabolism. The influence of alloxan on the uptake and metabolism of glucose in mammalian cells has been investigated using human diploid fibroblastic cells in culture, in order to elucidate the relation between alloxan action and insulin secretion in the β-cell. When monolayers of cells were exposed to radiolabelled glucose, the presence of alloxan (0.31-1.87 mM) resulted initially in a dose dependent enhancement of total cell glucose incorporation. This was observed within 2 minutes and declined by 6 minutes. After that time, alloxan inhibited glucose incorporation. When hexose transport was examined directly using glucose analogues, alloxan neither enhanced, nor inhibited the kinetics of uptake of 3-O-methyl-D-glucose or 2-deoxy-D-glucose. Alloxan (0.62-1.87) mM) exerted no measurable effect on cell permeability as determined by cell volume measurements using sucrose and D-mannitol. Thus alloxan appears to directly influence cell glucose metabolism beyond the level of phosphorylation. Alloxanic acid (0.62-1.87 mM), a decomposition production of alloxan, did not affect glucose incorporation in the fibroblasts. In addition, prior exposure of fibroblasts to alloxan (0.62-1.87 mM) for 6 minutes did not influence trypan blue exclusion by fibroblasts, nor impair ^{3}H-leucine incorporation in the fibroblasts. Analysis of cell extracts after cell monolayers were exposed to ^{14}C-U-D-glucose indicated that the initial stimulation by alloxan

was observed primarily in the nucleotide fraction (ribose) with inhibition of lactate production. The subsequent inhibition was observed in the nucleotide fraction. Assay of $^{14}CO_2$ production indicated that alloxan enhanced $^{14}CO_2$ formation from ^{14}C-U-D-glucose for approximately 10 minutes followed by inhibition. In order to probe the site of alloxan action, rates of $^{14}CO_2$ formation from 1- and 6-position labelled ^{14}C-glucose were compared. The initial stimulation was observed mainly in ^{14}C-1-D-glucose oxidation, whereas inhibition was measurable with the 6-positioned tracer. The results suggest that alloxan initially stimulates the pentose-monophosphate shunt and then subsequently inhibits both of the pentose-monophosphate shunt and the glycolytic-TCA pathway.

(g) Action of Pancreatic Hormones Other than Insulin on Cell Glucose Metabolism. The influence of somatostatin on cell glucose metabolism has been investigated using cultured human diploid fibroblasts. Over a wide range of concentrations somatostatin did not influence either hexose transport or total cell glucose utilization. The results indicate that acute changes in glucose metabolism induced by somatostatin in vivo are mediated directly through its effects on endocrine and gastrointestinal function.

(h) Studies of Glucose Metabolism and Insulin Action in Cultures from a Patient with Lipoatrophic Diabetes. Insulin binding and action of insulin on aspects of glucose metabolism have been investigated and compared to cultures from 9 nondiabetic controls. Incorporation of glucose was elevated in the patient's cells at glucose levels above 0.1 mM. When distribution of labelled glucose was examined, cell associated glycogen and acid soluble material were increased, but the greatest increment was in lactate production. Insulin binding, as indicated by maximum specific ^{125}I-insulin binding and concentration of unlabelled insulin for 50% displacement, was normal, although insulin regulation of the insulin receptor was less. The cells from the patient had reduced response of total glucose incorporation to insulin. When insulin stimulation of glycogen synthase was measured directly, the response to insulin was also attenuated. On the other hand, hexose transport levels and insulin stimulation of hexose transport appeared to be unimpaired. The data indicate alterations in both cell glucose metabolism and in response to insulin which may be related to the observed insulin resistance of this disorder.

(2) Comparison of insulin action in fibroblasts from diabetic and nondiabetic Pimas and Caucasian controls.

(a) Cell Growth. Cultures were derived from 19 subjects between the ages of 20 and 42 years. Seven were from Pimas with severe diabetic who had two diabetic parents and relatively early age of onset. Six were from Pima nondiabetics who had two nondiabetic parents. Six control cultures were established from Caucasian nondiabetics with no family history of diabetes. Cell density at confluence, plating efficiency, thymidine incorporation, and population doubling rates were monitored as a function of cell population doublings until the senescence of each culture. Fibroblast cultures from the Pima diabetics did not differ from those of the Pima nondiabetics in replicative life span or any of the measured culture parameters. When cultures

from the Pimas were compared with those of the Caucasian controls, however, they had decreased cell density at confluence, slower population doubling times, decreased thymidine incorporation, shorter replicative life spans, and lower plating efficiency. The data suggest that altered growth properties of cultures from diabetics may not be a requisite of diabetic status; they may reflect a genetic background that accompanies the expression of diabetes.

(b) Insulin Action. Insulin stimulation of hexose transport, glucose metabolism and glycogen synthase activity has been compared in fibroblasts from diabetics and nondiabetics. There were no differences in insulin sensitivity or magnitude of response in terms of total cell glucose utilization, hexose transport, or glycogen synthase activity. The data indicate there are no inherent differences in cellular responsiveness to insulin's glucoregulatory action in diabetics. The results of this study thus imply that the insulin resistance manifest in diabetics in vivo are not the cause, but more likely the consequency of the hyperglycemia.

Significance to Biomedical Research and the Program of the Institute: Resistance to insulin mediated glucose uptake is a well documented component of obesity and diabetes. It is not possible from in vivo studies, even if accompanied by measurements of freshly collected blood cells or tissues, to determine if the observed insulin resistance is a secondary response to the other dynamics of the hyperglycemic or obese state, or if it is a reflection of inherent cellular differences in response to insulin which contributed to the development of these disorders. Diploid fibroblast cultures established from skin biopsies afford an easily accessable system for the examination of human cellular metabolic capacity which is isolated from physiological variables. Our data to date indicate that the human fibroblast possesses a specific insulin receptor and that several points of carbohydrate metabolism respond to physiologic levels of insulin. The results demonstrate that this method is a powerful tool for the comparison of insulin sensitivity of cells derived from patients with disorders of insulin action. The availability of the Pima population is a further advantage. Extensive clinical and demographic data on several generations are available for selection of subjects, and the homogeneity of the population is an advantage in eliminating other variables which might confound the comparison. The results to date suggest the impaired insulin sensitivity observed in in vivo studies of individuals with abnormal glucose tolerance may not be due to inherent genetic differences in sensitivity to insulin action but rather to the complex physiological changes associated with the hyperglycemic state. Future studies combining measurements of insulin binding, receptor regulation, hexose transport and other intracellular responses to insulin, will lead to an understanding of the mechanism of insulin resistance and its cellular basis.

Proposed Course:

1. Studies of cell insulin action. We will continue to examine insulin stimulation of glucose metabolism in the fibroblast, in order to

further investigate the mechanism of insulin action on cell metabolism and to investigate variables which influence the cell response to insulin. These studies will include:

(a) Correlation of insulin binding and action. Insulin binding will be measured on monolayer cells and correlated with insulin action on glycogen aynthase and total cell glucose uptake. Effect of down regulation of the insulin receptor on insulin action will also be examined. This will yield data on insulin action when insulin binding and response are measured under identical conditions.

(b) Studies of insulin action on the pentose shunt and glycolytic pathway. Effect of insulin will be examined using 1 and 6 position labelled glucose and chromatographic analyses of the stimulated cells in order to determine which paths of glucose metabolism are influenced by insulin.

(c) Examination of the action of insulin analogs and mitogens on cell glucose metabolism. Insulin analogs and mitogens such as MSA will be compared in their actions on the parameters of glucose metabolism examined to date. The data will be correlated with binding data obtained in whole cell and isolated receptor fractions (see Z01 AM 54020-02-DD). Cell cultures fro the patient with lipoatrophic diabetes will be included in these studies in order to further examine the attenuated response observed in these cells.

(d) Examination of an intracellular intermediate of insulin action in the fibroblast. In coordination with Dr. Larner extracts of insulin stimulated cells will be assayed for the presence of a low molecular weight intermediate of insulin action observed previously in muscle and adipocytes. If present the chemical properties of the intermediate will be examined and the system will be used for study of time course and long term metabolism of this compound.

2. Comparison of diabetic and nondiabetic fibroblasts. Since no inherent differences in insulin action on glucose metabolism has been detected in diabetic cells, studies will center around the hypothesis that insulin resistance is induced by the hyperglycemia, hyperinsulinemia or other changes induced in the diabetic state. Therefore influence of glucose, insulin and other counterregulatory hormones will be examined on cell insulin action using the systems described above. In addition, since the fibroblast is not a quantitatively significant target tissue for in vivo insulin action, studies will be extended to the endothelium. The latter constitutes an important first site of plasma insulin action in vivo. Endothelium may be isolated from human sources (umbilical veins) and cultured in vitro. The systems for assay of insulin action which were developed for the fibroblast will be employed for examination of insulin action in this cell type. In addition, the availability of umbilical cords from diabetic mothers will allow comparative studies of diabetic and nondiabetic endothelium.

Publications:

1. Howard, B.V., Mott, D.M., Fields, R.M., and Bennett, P.H.: Insulin stimulation of glucose entry in cultured human fibroblasts. J. Cell. Physiol. 101:129-138, 1979.

2. Mott, D.M., Howard, B.V., and Bennett, P.H.: Stoichiometric binding and regulation of insulin receptors on human diploid fibroblasts using physiologic insulin levels. J. Biol. Chem. 254:8762-8767, 1979.

3. Howard, B.V., Fields, R.M., Mott, D.M., Savage, P.J., Nagulesparan, M. and Bennett, P.H.: Variation in growth characteristics of human diploid fibroblastic cell cultures corresponding to genetic origin rather than diabetic status. Diabetes 29:119-124, 1980.

4. Hidaka, H., Howard, B.V., Kosmakos, F., Fields, R.M., Craig, J.W., Larner, J.L., and Bennett, P.H.: Insulin stimulation of glycogen synthase in cultured human diploid fibroblasts. Diabetes (in press) 1980.

5. delaLlera, M., Rothblat, G., and Howard, B.V.: Cell triglyceride accumulation from very low density lipoproteins isolated from normal and hypertriglyceridemic human sera. Biochim. Biophys. Acta 574: 414-422, 1979.

6. Macarak, E.J., delaLlera, M., Kefalides, N.A., and Howard, B.V.: A comparison of the intracellular lipids of cultured vascular edothelial cells and fibroblasts. In Vitro 15:937-940, 1980.

7. Hara, H., Hidaka, H., Kosmakos, F., Mott, D.M., Howard, B.V., and Bennett, P.H.: Characterization of the human insulin receptor isolated from cultured fibroblasts and erythrocyte cell membranes. J. Clin. Endocrinol. Metab. (in press) 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54020-03 DD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of Insulin Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	M. Nagulesparan	Guest Worker	PCRS, NIAMDD
	B. Vasquez	NIH Postdoctoral Fellow	PCRS, NIAMDD
	P.H. Bennett	Chief	EFSB, NIAMDD

COOPERATING UNITS (if any)

Human Diabetes Study Center, University of Texas Health Science Center

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 95016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.8	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Regulation of insulin receptors is being studied in vivo using circulating mononuclear cells and in vitro using cultured diploid fibroblasts. Down regulation has been characterized for the fibroblast and this system has been used to detect inhibitors of insulin action in plasma from insulin resistant subjects. Investigation of monocytes and fibroblasts suggest that cells from a selected group of obese subjects have more receptors, reduced insulin affinity and decreased down regulation. Insulin binding to monocytes from obese glucose tolerant and intolerant subjects was the same, indicating that a source of insulin resistance distinct from the receptor is present in obese Pima subjects. Insulin receptors have been solubilized and partially purified from cultured human diploid fibroblasts and from human erythrocytes. Characterization of insulin binding to solubilized insulin receptor preparations resulted in binding affinity, pH optimum and analog specificity data similar to those for the intact cells.

PHS-6040

Project Description:

Objectives: Insulin binding in hyperinsulinemic non-diabetic Pimas is not suppressed below normal Caucasian levels, indicating there may be an abnormality in receptor regulation in this population. This failure of elevated insulin to decrease insulin binding could be due to a defect at the cellular level or to a circulating inhibitor. Our objectives include (1) to determine if the increased obesity of the Pima subjects can explain this apparent difference in receptor regulation, (2) to use the cultured fibroblast to investigate genetic differences in insulin binding and receptor regulation between diabetic and normal Pima and Caucasian subjects, (3) to use solubilized fibroblast insulin receptor preparations to examine structure and regulation of the insulin receptor and mitogen binding activity, (4) to study plasma from obese and diabetic subjects for their effect on in-vitro insulin receptor regulation. This last technique will be used to detect possible inhibitors of insulin action in plasma from insulin resistant subjects.

Methods employed and results:

Insulin binding to Pima and Caucasian Monocytes: ^{125}I-insulin binding was studied using circulating mononuclear cells from 19 normal Pima and 18 normal Caucasian subjects over a range of obesity. Both groups showed a weak negative correlation of specific insulin binding with fasting insulin. Body mass indices of the Pima subjects were correlated with the concentration of insulin required for half-maximum displacement of bound ^{125}I-insulin and with the high affinity K_D ($r=0.57$, $p<0.02$ and $r=0.73$, $p<0.0005$ respectively). Similar correlations were obtained for the affinity of Caucasian cells over a range of BMI. Because BMI and insulin are so closely correlated in these subjects ($r=0.80$, $p<0.0003$) it is not clear whether insulin or some other factor strongly associated with obesity is responsible for the observed variation in insulin receptor affinity. No significant differences were found between normal Pima and Caucasian monocyte insulin binding compared over a range of obesity.

The effect of caloric restriction and weight loss on insulin binding to monocytes from normoglycemic (n=8) and diabetic (n=7) subjects were studied. The diabetic and nondiabetic groups had a similar mean BMI and had insulin binding curves which were nearly identical prior to caloric restriction. Following caloric restriction both groups showed a similar magnitude of increased insulin binding over the 0.25 to 10^4 ng/ml range of insulin studied. This increase in insulin binding coincided with improved glucose tolerance in the diabetic group.

Pima subjects were studied to determine whether the insulin resistance of glucose intolerant obese subjects can be accounted for by obesity alone. Insulin resistance was assessed by measuring insulin-mediated glucose disposal during an infusion of a mixture of insulin, glucose, and somatostatin. Despite similar steady state plasma insulin levels, the mean steady state plasma glucose concentration was higher in the glucose intolerant group than in weight-matched subjects with normal glucose tolerance. Insulin binding to mononuclear cells was measured in samples from 8 glucose intolerant

[BMI=38±3 (range 30-54), mean fasting insulin 34±3 μU/ml, SSPG=220±11 mg/dl] and 9 normal subjects [BMI=37±3 (range 28-57), mean fasting insulin 24±3 μU/ml, SSPG=148±22 mg/dl]. The percent maximum specific binding per 8 x 10^6 monocytes per ml was 6.6±0.8 for the glucose intolerant subjects and 7.0±0.5 for the normal subjects (NS). There was no difference in insulin binding curves between the two groups.

Insulin binding to Pima and Caucasian Fibroblasts: A survey of insulin binding to diploid fibroblasts from seven Pima and six Caucasian subjects with normal glucose tolerance showed a correlation (r=0.78, p<0.002) between the high affinity K_D and the BMI of the cell donor. This decrease in affinity with increasing obesity appear to be accompanied by an increase in the high affinity receptor number with increasing BMI (r=0.66, p<0.02). A similar study of fibroblasts from five diabetic Pima subjects showed no correlation of binding parameters with BMI. This is in contrast to a strong correlation observed between the high affinity K_D and BMI (r=0.90, p<0.005) of fibroblasts from normal Pima donors.

Solubilized insulin receptor studies: Insulin receptors have been partially purified from erythrocytes and cultured human diploid fibroblasts. Cell membrane fractions were prepared by differential centrifugation and solubilized by Triton X-100. Gel filtration chromatography on Sephadex G-200 yielded a single peak of insulin binding activity with an apparent molecular weight of 370,000. Solubilized fibroblast receptor preparations showed pH optimum (pH=8.2) and analog specificity (porcine insulin > guinea pig insulin > proinsulin > desoctapeptide insulin) similar to those for whole cells. Scatchard analysis of insulin binding data for solubilized receptor and intact fibroblasts yielded nearly identical high affinity association constants (Ka) of 1.8 x 10^9M and 1.3 x 10^9M, respectively. The average affinity profile analysis of the same data yielded limiting high affinity association constants (Ke) of 7.1 x 10^8M and 1.3 x 10^8M, respectively. The solubilized insulin receptor isolated from erythrocytes and fibroblasts displayed very similar parameters of insulin binding and the molecular weights of the receptors were the same. The similarity of the characteristics of the insulin receptor solubilized from these two sources provides evidence against significant modification of the fibroblast insulin receptor by the brief trypsin treatment used to release the cells from the monolayers.

In vitro insulin receptor regulation in fibroblasts: Maximum insulin mediated reduction in insulin binding (MIR) to diploid fibroblasts from diabetic and non-diabetic Pima Indians and non-diabetic Caucasians was compared for the determination of possible genetic differences in these cells. The mean maximum specific binding per cell was the same in a comparison between 7 diabetic Pimas, 8 normal Pimas and 7 normal Caucasian lines. The mean MIR was also not significantly different for the three groups (58±13%, 52±16% and 64±14% respectively). When the Pima subjects were subdivided by the degree of obesity [i.e. BMI <30 (n=3), BMI >39 (n=5)] the more obese Pima subjects showed a reduced mean MIR (43±14% v.s. 65±5%).

Detection of an insulin resistance related plasma factor: Plasma from a 32 yr old male with congenital lipoatrophic diabetes (LD) with documented severe insulin resistance has been studied for its effects on insulin mediated down regulation of fibroblast insulin receptors. Percent down regulation was calculated from the amount of insulin binding/cell following a 16 hour, 37°C incubation of normal diploid fibroblast monolayers in medium with 20% plasma or 0.1% bovine serum albumin (BSA) and varied insulin concentrations. Maximum down regulation in the presence of LD plasma was significantly reduced compared to the mean obtained using plasma from 4 normal subjects (37±6% v.s. 60±5%, $p<0.001$). When compared to normal plasma over a range of insulin concentrations, LD plasma caused a significant reduction in DR at 2.5 ($p<0.002$), 10.0 ($p<0.001$) and 100 ($p<0.008$) ng/ml insulin. The concentration of insulin required for ½ maximal down regulation was increased in the presence of the LD plasma (2.0±0.1 for normal v.s. 8.0±0.8 ng/ml for LD). 20% plasma from normals or LD had no effect on maximum specific insulin binding/cell when compared to preincubation of cells with 0.1% BSA. Normal plasma had no significant effect on DR when compared with BSA over the range of insulin concentrations studied. LD plasma did not differ from normals when tested for insulin degradation and did not contain antibodies against insulin or the insulin receptor. We conclude that 1) LD plasma contains factor(s) which alter the sensitivity and magnitude of insulin mediated down regulation; 2) insulin mediated down regulation of insulin receptors can be used to detect plasma factors which may contribute to the insulin resistance of Pima Indians.

Significance to Biomedical Research and the Program of the Institute: Initial studies of insulin binding to Pima monocytes suggested that the level of binding was normal but the in vivo regulatory effect of insulin on receptor number was not the same as observed for Caucasians. Our recent results indicate that Caucasian and Pima monocytes in vivo and fibroblasts in vitro respond similarily to insulin to reduce insulin binding. Also both races and cell types show an effect of BMI on receptor affinity. The apparent altered receptor regulation initially observed for hyperinsulinemic Pima subjects may have been confound by the increased obesity of the Pima compared to the Caucasian subjects. Preliminary results comparing receptor regulation in fibroblasts indicate reduced down regulation of insulin receptors from obese donors but comparable receptor regulation for normal weight Pima and Caucasian donors. These results suggest the importance of genetic factors which must be considered in interpretation of insulin binding and receptor regulation for obese subjects.

The methods developed to prepare solubilized insulin receptors from fibroblasts provide a tool to further study alterations in receptor structure and regulation in obese subjects. Since several effects of insulin action on cultured fibroblasts have been described by this and other laboratories the potential also exists for the coordinated study of insulin receptor biochemistry and insulin action.

The results of studies on the lipoatrophic diabetic indicate the presence of a plasma factor(s) which inhibits insulin action. In addition, these results

demonstrate a technique which can be used to screen plasma from other insulin resistant subjects for factors which alter insulin action.

Proposed Course:

Insulin binding to Pima and Caucasian fibroblasts and monocytes. The decreased insulin affinity observed for obese Pima subjects may be the result of an alteration in 1) receptor structure, 2) negative co-operativity or 3) composition and/or structure of the cell membrane. A comparison of the parameters of insulin binding using purified receptors from obese and thin Pima fibroblast donors will determine the importance of receptor structure for this phenomena. Possible differences in negative cooperativity between fibroblast insulin receptors from obese and thin subjects will be examined by comparing ^{125}I-insulin dissociation kinetics using the method of DeMeyts.

Solubilized receptor studies. The methods used to isolate the membranous fraction of the homogenized fibroblasts will be modified to exclude insulin receptors originating from intracellular structures (golgi, lysosomes). A two-phase polyethylene glycol/dextran system will be used to prepare fibroblast plasma membrane prior to solubilization of the receptors. Marker enzyme activities, including Na^+/K^+ ATPase, will be monitored to assess plasma membrane purification. Binding of insulin and the mitogen, MSA, will be compared in whole cells, plasma membrane and solubilized receptor preparations.

In Vitro insulin receptor regulation in fibroblasts. Insulin receptor regulation studies on fibroblasts from additional obese and thin normal Pima donors are needed to clarify a possible distinction in their extent of MIR. The observation that cells from obese subjects have reduced receptor affinity and possibly increased receptor numbers suggests that regulation of their insulin binding may also be different. Scatchard analysis of insulin binding following receptor regulation using fibroblasts from obese and thin donors will indicate if both groups reduce their receptor numbers or their affinity.

Detection of an insulin resistance related plasma factor. The technique used to demonstrate a plasma factor which inhibits insulin mediated down regulation will be used to screen plasma from normoglycemic and glucose intolerant Pima subjects. The detection of such a factor will initiate purification and characterization studies of the inhibitor.

Publications:

Mott, D.M., Howard, B.V., Bennett, P.H.: Stoichiometric binding and regulation of insulin receptors on human diploid fibroblasts using physiologic insulin levels. J. Biol. Chem. 254: 8762-8767, 1979.

Savage, P.J., Bennion, L.J., Flock, E.V., Nagulesparan, M., Mott, D.M., Roth, J., Unger, R.H., Bennett, P.H.: Diet-induced improvement of abnormalities in insulin and glucagon secretion and in insulin receptor binding in diabetes mellitus. J. Clin. Endocrin. Metab. 48:999-1007, 1979.

Nagulesparan, M., Savage, P.J., Mott, D.M., Johnson, G.C., Unger, R.H., and Bennett, P.H.: Increased insulin resistance in obese, glucose intolerant Southwestern American Indians: Evidence for a defect not explained by obesity. J. Clin. Endocrin. Metab., In press 1980.

Hara, H., Hidaka, H., Kosmakos, F.C., Vasquez, B., Mott, D.M., Howard, B.V., and Bennett, P.H.: Characterization of human insulin receptor solubilized from cultured fibroblast and erythrocyte cell membrane preparations. J. Clin. Endocrin. Metabo., In press 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54021-03 DD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Hemoglobin A1 Levels in Diabetes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	W.C. Knowler	Chief	SFSS, NIAMDD
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LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.5	0.4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Glycosylated hemoglobin (HbA_1) concentrations were measured in 600 Pima Indians, aged 15 to 91 years, who were participating in the epidemiological studies of diabetes in the Gila River Indian Reservation in Arizona. At the same time plasma glucose levels, fasting and two-hours post-glucose load (75 g), were determined and the plasma glucose values were compared with HbA_1 levels in the same population to assess the utility of HbA_1 measurements in the diagnosis of diabetes. The distribution of glucose levels in Pimas has been described previously by a model of two overlapping Gaussian curves. Attempts were made to fit the same model to the HbA concentrations. For patients aged 25 years and over, the frequency of HbA_1 levels were bimodally distributed and, like the glucose levels, satisfactorily fit the overlapping Gaussian distribution model. These observations indicate the presence of two subpopulations in the Pima Indians, one normal and one diabetic. HbA_1 levels in the nondiabetic portion of the population were found to increase as a function of age in the Pimas, as expected. Levels of HbA_1 were highly correlated with both fasting and two-hour post-load plasma glucose values, but did not discriminate better than the glucose data between diabetic and nondiabetic subjects.

Project Description:

Objectives: The present study was designed to determine the feasibility of using glycosylated hemoglobin measurements to examine the relationship between vascular complications and hyperglycemia. Glycosylated hemoglobin levels in the Pima population were examined to determine if they could 1) discriminate between the diabetic and non-diabetic groups within a population, and 2) correlate with microvascular complications.

Methods Employed: Glycosylated hemoglobin (HG A1) was measured by a modification of the method of Trivelli, involving washing the red cells with physiologic saline, hemolyzing the cells with water and toluene, dialyzing the hemolyzate against a cyanide-phosphate buffer and chromatographing the hemolyzate on a Bio-Rex 70 column. The glycosylated hemoglobin was eluted with a cyanide-phosphate buffer (pH 6.7) and then the rest of the hemoglobin was eluted with a phosphate buffer (pH 6.4). The optical density of the two fractions was measured at 415 nm and the percentage of the total hemoglobin that was glycosylated was calculated. The frequency distributions of glycosylated hemoglobin in the 15 to 24 and 25 and over age groups were examined and compared with the fasting and two-hour glucose concentrations and with the incidence of retinopathy. Maximum likelihood methods were used to determine the mean and standard deviations of the hemoglobin distributions.

Major Findings: A single distribution of glycosylated hemoglobins was found in the young males, aged 15 to 24 years. In the young females, a single mode was seen corresponding to the normal population with a few outlying values indicating the presence of a small number of diabetics in this age group. In those age 25 and over in both males and females the hemoglobin levels were bimodally distributed with a satisfactory fit to the overlapping Gaussian distribution model previously applied to the frequency distribution of plasma glucose levels in the Pima population. The characteristics of the nondiabetic components of the HbA_1 distributions in 5 age groups (15-24-34-44-54 and 55+ years) were examined. The mean HbA_1 concentration in the 15-24 year age group averaged 10.3+0.9% (SEM) but increased significantly with age at a rate 0.25% per decade. The diabetic component had mean levels averaging 17.5%. The cut-off points between the normal and diabetic components ranged from 13.5 to 14%. Distinct elevation of glycosylated hemoglobin levels did not occur until fasting hyperglycemia ($\geq$140 mg/dl) was present. Glycosylated hemoglobin levels are highly correlated with both fasting and post-load plasma glucose levels and will not discriminate better than glucose measurements between non-diabetic and diabetic subjects. Triglyceride concentrations showed only low order correlations with glycosylated hemoglobin levels.

Significance to Biomedical Research and the Program of the Institute: The presence of two subpopulations in the Pima Indians, one normal and one diabetic, is clearly indicated by the frequency distributions of glycosylated hemoglobins, fasting glucose levels and two-hour glucose levels. The determination of glycosylated hemoglobin can be useful for epidemiological studies where the performance of glucose tolerance testing is impractical.

Proposed Course: This project will be continued to further characterize the association between hemoglobin A1 levels and the prevalence and incidence of diabetic complications. In addition, HbA_1 levels will be followed in longitudinal studies of individual patients and assessed as an indication for prediction of development of diabetes and its complications.

Publications:

Flock, E.V., Bennett, P.H., Savage, P.J., Webner, C.J., Howard, B.V., Rushforth, N.B., and Miller, M.: Bimodality of Glycosylated Hemoglobin Distribution in Pima Indians: Relationship to Fasting Hyperglycemia. Diabetes 28: 984-989, 1979.

Bennett, P.H., Flock, E.V., and Rushforth, N.B.: Bimodality of Glycosylated Hemoglobin Distributions in the Pima Indians: Evidence that Nondiabetic Levels Increase with Age. Excerpta Medica: 10th Congress of the International Diabetes Federation. No. 481: 20, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54023-03 DD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Very low density lipoprotein metabolism in Pima Indians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.V. Howard	Assistant Chief	PCRS, NIAMDD
Other:	J.S. Reitman	Staff Physician	PCRS, NIAMDD
	S.L. Aronoff	Chief	PCRS, NIAMDD
	L. Zech	Staff Physician	IRMD, NHLBI

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Scott M. Grundy, M.D., Dept. of Medicine, School of Medicine, Univ. of CA, San Diego, CA
Maria-Riita Taskinen, M.D., Dept. of Medicine, Univ. of Helsinki

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.5	0.8

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pima Indians have a high prevalence of obesity, diabetes mellitus and hyperinsulinemia, but they have low plasma cholesterol levels, reduced LDL synthesis, and greatly decreased incidence of cardiovascular disease. VLDL metabolism in Pima Indians will be investigated in order to further investigate lipoprotein metabolism in this population. Synthetic and catabolic rates for VLDL will be compared in groups having different insulin levels using tritiated glycerol as a precursor. VLDL metabolism will be studied in a group diabetics to determine the influence of hyperglycemia on VLDL metabolism in this population. Conversion of VLDL to LDL will be studied using ^{125}I VLDL and ^{131}I LDL simultaneously. Postheparin, muscle, and adipose tissue lipoprotein lipase levels will be measured. The results of this study should yield data on VLDL metabolism and its conversion to LDL in this population and explore the relation between these lipoproteins and cardiovascular disease.

Project Description:

Objectives:

1. The metabolism of VLDL will be studied using a pulse injection of ^{3}H glycerol. This will yield values for synthetic and catabolic rates of VLDL which can be compared to those of Caucasians assayed under similar conditions.

2. The relation between plasma insulin levels and VLDL synthetic and catabolic rates will be determined by ^{3}H glycerol VLDL studies in Pimas having differing serum insulin levels.

3. VLDL metabolism will be studied in a group of untreated male diabetics matched in age and weight to group #1. Effect of hyperglycemia on synthetic and catabolic parameters will be assessed.

4. The effect of exercise on VLDL metabolism in Pimas will be studied using ^{3}H glycerol.

5. The conversion of VLDL to LDL will be studied in a group of Pima Indians using I^{125} and I^{131} LDL. This will yield an independent measure of VLDL metabolic rates, and also will measure the extent of conversion of VLDL to LDL in this population.

6. Plasma free fatty acids, which are the immediate precursors of the fatty acids in VLDL triglyceride, will be quantified and turnover will be assessed by a pulse injection of albumin bound ^{14}C free fatty acid. Free fatty acid levels and turnover rates will be compared in groups having low and high insulin levels to determine if adipose tissue free fatty acid production reflects or correlates with fasting serum insulin values.

7. Post heparin lipolytic activity and adipose and muscle lipoprotein lipase activities will be determined in a number of Pima Indians over a range of obesity to determine if VLDL metabolism in the Pimas is related to the levels of this enzyme. Observations will be made on the effects of exercise and weight loss on lipoprotein lipase activity.

Major Findings:

1. ^{3}H glycerol studies. VLDL metabolism was studied, using ^{3}H-glycerol as an endogenous precursor of triglyceride synthesis, in 15 obese Pima nondiabetic males and compared to that of 20 obese and 13 normal weight, normolipidemic, nondiabetic Caucasian males. The resultant kinetic data were analyzed using a multicompartmental model which includes two pathways for VLDL-TG synthesis and a process of stepwise delipidation for VLDL catabolism. As compared to obese Caucasians the obese Pimas had a lower rate of VLDL-TG synthesis, and a lower proportion of slow pathway for synthesis. The fractional catabolic rate in the Pimas was higher than in either Caucasian group. When the relation between VLDL-TG metabolism and plasma insulin was examined,

plasma insulin levels in the Pima were not correlated with VLDL-TG synthetic rates, catabolic rates or plasma pools. On the other hand VLDL-TG synthetic rates were correlated with plasma free fatty acid levels. Thus, in the Pima populatin, VLDL-TG synthesis is low, VLDL-TG catabolism is accelerated, and VLDL pools appear to be insensitive to the influence of body weight and hyperinsulinemia.

The kinetic data on VLDL-TG observed from the Pima revealed increased fraction delipidized, a short VLDL residence time and a low proportion converted to IDL; the relation between these observations and VLDL particle size must be explored. Finally, there was a low proportion of slow/fast pathway for VLDL-TG production in all the Pimas studied, and this parameter was elevated in the more obese subjects of both Pima and control populations. Interpretation of this awaits further elucidation of the nature of the two pathways of VLDL-TG production.

Tc date 8 untreated diabetics have been studied, and preliminary analysis of the data suggest hyperglycemia may be accompanied by changes in both synthesis and catabolism of VLDL.

The IDL fraction (density 1.006-1.019) has been isolated from 8 of the non-diabetic patients, and the specific activity data from this fraction is being used for the development of a multicompartmental model of IDL metabolism.

2. ^{125}I studies and free fatty acid metabolism. No patients have been admitted for these objectives.

3. Lipoprotein lipase studies. In collaboration with Drs. Taskinen and Nikkila we have measured adipose tissue, muscle and post-heparin lipoprotein lipase activity in 10-12 Pima Indians with a wide spectrum of obesity. LPL activity expressed per mg wet weight of tissue (fat and muscle) or as total heparin-releasable activity was not correlated with percent of IBW in the subjects. Data will also be expressed per cell number, since other studies have shown a relationship between adiposity and this enzyme when expressed as activity per adipose cell.

Significance to Biomedical Research and the Program of the Institute: The results of these studies are relevant to the understanding of in vivo lipoprotein metabolism, and its relationship to diabetes. The studies on the interconversions of VLDL to LDL will determine why LDL synthesis is low in the Pimas. The comparison of VLDL metabolism in populations with high and low insulin levels will indicate whether or not increases in serum insulin lead to increased synthesis of VLDL. Furthermore, a comparison of VLDL and LDL turnover in Caucasians with that of the Pimas will yield information on the relationship between lipoprotein turnover and cardiovascular diseases.

The findings of elevated VLDL-FCR in obese, non-diabetic Pima Indians raise the question of whether this key lipogenic enzyme is instrumental in mediating the observed increased FCR. In addition, higher adipose LPL levels may be important in the etiology of the obesity observed in the Pima population.

The measurment of LPL before and after weight loss will yield information on the role of this enzyme in obesity.

It is known that in normal subjects muscle LPL levels increase with physical training; if adipose tissue LPL levels increase less or even decrease, then exercise training provides a mechanism whereby muscle lipogenesis is increased at the expense of adipose tissue lipogenesis. The measurements for VLDL metabolism and lipoprotein lipase before and after exercise will test this hypothesis.

Proposed Course:

^{3}H Glycerol studies. Studies will continue of VLDL-TG metabolism in a group of male diabetic Pima Indians matched in age and weight to the previous group of nondiabetics. Diabetics will be untreated with 2 hour plasma glucose levels greater than 250 mg/dl. Data will be analyzed to determine the influence of hyperglycemia on VLDL-TG metabolism in this population.

Isolation of IDL fractions will continue and specific activity data used for further development of an IDL model and analyses of IDL metabolism in this population.

To investigate the mechanism of the decreased blood VLDL levels observed with exercise, we will study VLDL metabolism before and after exercise training. These studies will be accompanied by before and after measurements of muscle and fat LPL activities. This will determine whether changes in VLDL-TG synthesis or FCR are responsible for the decrease in VLDL-TG pools after exercise.

Studies of objectives 4 & 5: These will await the completion and analyses of the ^{3}H glycerol studies.

Studies of lipoprotein lipase: Studies will be continued to determine muscle, adipose and post-heparin lipoprotein lipase activity in Pimas and Caucasians over a wide range of obesity. Enzyme measurements will be performed by Drs. Taskinen and Nikkila and results will be correlated with weight, measurements of adipocyte cell number and plasma lipid values.

Adipose, muscle and post-heparin lipoprotein lipase levels will then be measured in a group of obese Pima Indians before and after weight reduction to determine the response of the enzyme in these tissues to decreased fat depots.

It has been established that acute exercise of sufficient severity or duration can result in decreased blood triglyceride levels, and the effect is cumulative over several days of repeated exercise. This occurs presumably because of triglyceride uptake either during the exercise (for use by muscle tissue) or during subsequent rest period for replenishment of muscle and/or adipose tissue TG stores utilized during exercise. We plan to measure tissue and post-heparin lipase levels before and after a period of exercise and relate these to the results of VLDL-TG metabolism determined during the same

periods.

Publications:

Howard, B.V., Zech, L., Davis, M., Bennion, L.J., Savage, P.J., Nagulesparan, M., Bilheimer, D., Bennett, P.H., and Grundy, S.M.: Studies of Very Low Density Lipoprotein Triglyceride Metabolism in a Population with Low Plasma Lipids - Lack of Influence of Body Weight or Plasma Insulin. J. Lipid Res., in press 1980.

Howard, B.V., Zech, L., Davis, M., and Grundy, S.M.: Very Low Density Lipoprotein Metabolism in the Pima Indians. In Proceedings of the Workshop on Lipoprotein Kinetics. Eds.: M. Berman et al. Academic Press, NY, in press 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54024-02 DD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

In-Vitro Insulin Resistance in Pima Indians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.L. Aronoff	Chief	PCRS, NIAMDD
Other:	F.C. Kosmakos	Visiting Scientist	PCRS, NIAMDD
	D.M. Mott	Research Biochemist	PCRS, NIAMDD
	B.V. Howard	Assistant Chief	PCRS, NIAMDD
	S. Cushman	Associate Chief	CMOS, NIAMDD
	L. Salans	Associate Director	DEM, NIAMDD

COOPERATING UNITS (if any)

Joseph Larner, U. of Virginia Medical School, Charlottesville, VA

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The marked insulin resistance observed in the Pima Indians could be the result of a defect at the level of insulin, the insulin receptor, metabolic pathways, or any combination of the above. By a variety of sensitive criteria, the defect does not appear to be due to insulin or the insulin receptor. The isolated adipocyte is being used as a model to detect the existence of insulin resistance in pathways of carbohydrate or lipid metabolism. Further, the degree of hyperplasia or hypertrophy in obesity is being assessed in the Pima Indians by measurement of total body adiposity and adipose cell number and cell size.

Project Description:

Objectives:

1. Characterization of the Pima Indian insulin receptor. The marked insulin resistance noted in the Pima Indians could result from a defect in either the insulin structure, the insulin receptor, a metabolic pathway, or a combination of the above. The Pima Indian insulin receptor will be characterized by various binding criteria and compared to studies with Caucasian cells. These studies will be performed with erythrocytes.

2. The role of the insulin receptor in mediating insulin resistance in Pima Indians. Alterations in insulin receptor number and affinity, both in vivo and in vitro, have been documented in instances of marked insulin resistance. Receptor number and affinity will be measured in Pima Indians with a wide range of fasting insulin levels to determine the role of the insulin receptor in mediating insulin resistance.

3. Comparison of insulin receptors on monocytes and erythrocytes. Alterations in insulin receptor number have been documented using circulating monocytes. The assay for monocytes requires 200-500 ml of blood, while similar studies with red cells can be performed with as little as 10 ml of blood. Parameters of insulin binding will be compared between monocytes and red cells from Pima Indians with a wide range of fasting insulin levels.

4. Characterization of metabolic pathways in isolated adipocytes from Pima Indians with insulin resistance. The isolated adipocyte provides a model system to measure insulin response in a major insulin target tissue. Parameters of carbohydrate and lipid metabolism will be compared in adipocytes from diabetic and nondiabetic patients over a wide range of obesity.

5. Cellular characterization of obesity in Pima Indians. An increase in fat mass is associated with either an increase in adipose cell number or adipose cell size. The role of cell number and size in the development of obesity in the Pimas will be assessed by measuring total body fat and total adipose cell number and size.

Methods:

1. Parameters of insulin binding will be measured on erythrocytes, monocytes and adipocytes using ^{125}I-insulin.

2. Carbohydrate metabolism of adipocytes will be assessed by measuring ^{14}C-glucose oxidation to $^{14}CO_2$ and glucose transport using ^{14}C-2-deoxyglucose.

3. Lipid metabolism of adipocytes will be monitored by:

   a. B-adrenergic stimulation of lipolysis (glycerol release);
   b. Insulin inhibition of B-adrenergic stimulation of lipolysis

4. Adipose cell number and cell size will be measured on osmic acid (2%) fixed cells using a coulter counter coupled to a cell sizer.

5. Total body fat will be indirectly measured by estimating lean body mass with D_2O.

Major Findings:

Insulin receptors on erythrocytes and monocytes. The insulin receptor of Pima erythrocytes is similar to Caucasian erythrocytes by various parameters of insulin binding, i.e., time and temperature dependence of binding, pH dependence of binding, and analogue specificity. The number of insulin receptors per cell did not correlate with the level of fasting insulin (10-61 μU/ml) when measured using either erythrocytes or monocytes. When insulin receptor affinity was measured in patients with various degrees of hyperinsulinemia, a negative correlation was observed between red cell receptor affinity and fasting insulin levels. This correlation was not detected in monocytes. Neither insulin receptor number nor affinity was correlated when compared between erythrocytes and monocytes of the same subject. These results suggest that the red cell and monocyte are not comparable systems for assessing receptor status. Insulin binding to erythrocytes was strongly dependent on the age of the red cell preparation. Binding was higher in the young cells and lower in the other cells. This decrease in insulin binding to erythrocytes with increase in cell age was due to a decrease in receptor number.

Fat cell studies. Studies with Pima adipocytes have just begun and no results are presently available.

Proposed Course: Total body adiposity, adipose cell number and cell size will be measured in Pimas with various degrees of obesity. These measurements will be made in conjunction with studies of insulin sensitive parameters of carbohydrate and lipid metabolism in adipocytes derived from diabetic and nondiabetic Pima Indians. Since the insulin receptor does not appear to contribute to insulin resistance it is anticipated that studies involving fat cells from Pima Indians will help to elucidate the mechanisms of insulin insensitivity found in these people.

Publications:

Kosmakos, F.C., Nagulesparan, M., and Bennett, P.H.: Insulin Binding to Erythrocytes: A Negative Correlation with Red Cell Age. J. Clin. Endocrin. Metab. In press, 1980

Kosmakos, F.C., Snowman, A., and Nagulesparan, M.: Characterization of the Pima Indian Insulin Receptor. Am. J. Physiol. Endocrin. Metab., In press, 1980

NIH Contract No: NO1-AM-6-2219 Starting Date: September 1, 1979
Expiration Date: August 31, 1980

Contractor: University of Texas Health Science Center
Southwestern Medical School

Money Allocated: $1,699,947.00 Man-Years Purchased: 23.7

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54 025-02 DD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

($Na^+ + K^+$)ATPase Activity in Obese Subjects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David M. Mott	Research Biochemist	PCRS, NIAMDD
Other:	Barbara V. Howard	Assistant Chief	PCRS, NIAMDD
	Peter H. Bennett	Chief	EFSB, NIAMDD
	Barbara Vasquez	NIH Postdoctoral Fellow	PCRS, NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Digestive Disease Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.5	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Erythrocyte ($Na^+ + K^+$)ATPase activity has been measured in Caucasian and Pima Indian subjects. Obese Pima subjects have a significantly altered Mg^{++} dependence for enzyme activity compared to thin Pima or Caucasians. Additional studies will determine the importance of this lesion as a genetic factor which promotes obesity. For this purpose, the activity will also be measured in diploid fibroblasts from thin and obese Pima Indian and Caucasian subjects.

Project Description:

Objectives: Pima Indians are a genetically homogeneous group characterized by a high incidence of obesity and hyperinsulinemia. Although a genetic basis for this obesity has not been established, the ob/ob mouse may serve as an animal model for Pima obesity. The ob/ob mouse has been found deficient in basal and thyroid inducible (Na^+, K^+)ATPase. The importance of this enzyme activity has been established for the thyroid stimulation of oxygen consumption. A deficiency of (Na^+, K^+)ATPase activity could make more calories available for stored fat since less energy is expended on ATPase activity. A deficiency in the induction of a single enzyme therefore, may explain the obesity of the ob/ob mouse. If this explanation for obesity applies to humans, obese subjects with normal thyroid function should have decreased plasma membrane (Na^+, K^+)ATPase activities and a diminished response of this enzyme to triiodothyronine. In-vitro basal and triiodothyronine stimulated (Na^+, K^+)ATPase activities will be compared in erythrocytes from thin and obese subjects with normal thyroid function. Results of this study will be used to assess the extent that an abnormal regulation of energy balance is responsible for obesity in Pima and weight matched Caucasian subjects.

Methods: (Na^+, K^+)ATPase activity is determined in erythrocytes as the ouabain sensitive release of inorganic phosphate.

Major Findings: (Na^+, K^+)ATPase activity per grams of hemoglobin has been measured in red cells from normal Caucasians and normal and obese Pimas. (Na^+, K^+)ATPase activity measured over a range (0.3 - 1.2mM) of Mg^{++} concentrations in 0.5 mM EDTA indicates a sharp peak at about 0.90 mM Mg^{++} for thin (BMI < 24) Caucasian (n=7) or Pima (n=4) subjects. The peak activity for obese (BMI > 30) Pima subjects however is shifted to a Mg^{++} concentration which is about 0.1mM lower. Comparing (Na^+, K^+)ATPase activity in thin and obese subjects, it is elevated for the thin ($p<0.02$) at 9 mM Mg but reduced at 0.6 mM Mg ($p<0.04$). A similar sharp Mg^{++} dependence curve for (Na^+, K^+) ATPase has been observed for diploid fibroblasts.

Significance to Biomedical Research and the Program of the Institute: The demonstration of altered red cell (Na^+, K^+)ATPase activity in obese Pimas compared to thin Pimas and Caucasians suggests that a genetic lesion effecting enzymatic hydrolysis of ATP could contribute to the excessive obesity in the Pima population. Studies on additional red cell samples as well as other tissues will indicate the prevalence of this abnormality in the Pima population and its casual relationship with obesity.

Proposed Course:

1. Red cell (Na^+, K^+)ATPase activity will be determined on 60 euthyroid subjects divided equally among obese and thin Pimas and Caucasians. Activity dependence on Mg^{++} concentration will be measured in laked red cells and red cell ghost preparations. Results from this study will clarify the association of altered (Na^+, K^+)ATPase Mg requirements with obesity.

2. Uptake of ouabain suppressible $^{86}Rb^{+}$ by red cells from Pima subjects will be studied to see if the altered activity observed for laked red cells is expressed as decreased (Na^{+}, K^{+}) pump activity in the intact erythrocyte. In addition, red blood cells from young pre-obese Pima subjects can be studied to determine if the observed altered ATPase activity is a result of the obese state or a possible cause.

3. The (Na^{+}, K^{+})ATPase activity will be measured in diploid fibroblasts from thin and obese Pima and Caucasian subjects. A comparison of this activity in a variety of cells cultured in a uniform environment should clarify possible genetic associations with obesity.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54026-01 DD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chlorpropamide-Alcohol Flushing - A predictor for non-insulin dependent diabetes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.L. Aronoff	Chief	PCRS, NIAMDD
Other:	M. Nagulesparan	Guest Worker	PCRS, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.1	0.6

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The CPAF test has been reported to be associated with non-insulin dependent diabetes. We therefore used this test to study the Pima Indian population a group with the highest reported prevalence of Type II diabetes in the world. Our findings so far suggest, that the Pima Indian subjects can "flush" with alcohol alone. In the small group studied so far there appears to be no clear association between Type II diabetes and the CPAF test. Studies are in progress to determine the validity of using the CPAF test an a predictor or test for Type II diabetes in the Southwestern American Indians.

Project Description:

Objectives: Some diabetics taking chlorpropamide have an intense facial flush after alcohol. This chlorpropamide induced alcohol flushing (CPAF) is reported to be a dominantly inherited trait associated with non-insulin dependent diabetes. It is much less frequent in insulin dependent diabetics and non-diabetics. CPAF has also been detected before the onset of glucose intolerance and therefore may possibly be useful as a genetic marker for some cases of non-insulin dependent diabetes. The nature of the association between CPAF and non-insulin dependent diabetes is not clear but has been suggested to be due to increased sensitivity to endogenous opiates such as enkephalin.

The Pima Indians appeared to be an excellent group to further examine the CPAF test since they are a homogeneous population with the highest reported prevalence of non-insulin dependent diabetes in the world. Further, since 50% of adult Pimas have diabetes and a non-diabetic Pima Indian with two diabetic parents (genetic prediabetic) has approximately 5 times the risk of developing diabetes as a nondiabetic Pima with two nondiabetic parents, the genetic prediabetic Pima Indian is an ideal subject to determine the predictive capability for diabetes of this test.

Purpose of Study:

1. To determine the relationship between CPAF and non-insulin dependent diabetes.

2. To determine the usefulness of CPAF as a predictor of non-insulin dependent diabetes by studying the genetic prediabetic Pima Indian.

Methods: Pima Indian subjects with normal glucose tolerance, impaired glucose tolerance and diabetes are being studied. On the first day, the subjects are given 40 ml of sherry over one minute and the skin temperature at the face and wrist measured for 60 minutes. Then, on the evening before the CPAF test, chlorpropamide (250 mg) is given orally. The following morning the subjects drink 40 mls of sherry over one minute (12 hours following the ingestion of chlorpropamide). Skin temperature is measured at the same location (2 cm below the outer canthus of the eye and 8 cm proximal to the wrist on the flexor aspect of the forearm). An increment of skin temperature of >2°F is considered a "flush".

Results: Preliminary studies demonstrate that some of the Pima Indian subjects had temperature increases greater than 2°F with alcohol (sherry) alone. In many subjects the temperature increase on the face (with alcohol or alcohol and chlorpropamide) was associated with temperature increases on the wrist as well.

Because of the above findings the study was expanded. The test was repeated at -12 and -6 hours before the ingestion of the chlorpropamide tablet, and at +12, +18 and +36 hours after the tablet. Thirty-two subjects (11 with

normal glucose tolerance, 8 with impaired glucose tolerance and 13 with diabetes) have been studied. The findings suggest (1) about 25% of the subjects have facial "flushing" with alcohol alone and another 25% with alcohol and chlorpropamide; (2) in more than half the subjects a facial flush was associated with a similar change in temperature on the wrists, indicating that there was a generalized increase in skin temperature and not a localized facial flush; (3) in this small group investigated there appears to be no clear association between the CPAF test and the diabetic status of the patient.

Proposed Course: So far the results of this study are inconclusive. We therefore propose to study more subjects to determine if the CPAF test can be used as a test or predictor of Type II diabetes in the Southwestern American Indian population.

Publications:

The findings of this study were presented at a workshop on "Chlorpropamide-Alcohol Flushing and Diabetes" sponsored by the National Diabetes Data Group of the N.I.H. and the International Diabetes Epidemiology Group., June 13-14, 1980 in Washington, D.C.

NIH Contract No: NO1-AM-6-2219 Starting Date: September 1, 1979
Expiration Date: August 31, 1980

Contractor: University of Texas Health Science Center
Southwestern Medical School

Money Allocated: $1,699,947.00 Man-years Purchased: 23.7

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54027-01 DD

PERIOD COVERED
February 1, 1980 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Effect of HGH Therapy on Somatostatin & Pancreatic PP & Lipoprotein Levels in GH Deficient Subjects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.L. Aronoff	Chief	PCRS, NIAMDD
Other:	B.V. Howard	Assistant Chief	PCRS, NIAMDD
	M. Nagulesparan	Guest Worker	PCRS, NIAMDD

COOPERATING UNITS (if any) R. Gingerich, Dept. of Pediatrics, Washington Univ. School of Medicine, St. Louis, MO; R.H. Unger, Human Diabetes Study Center, Univ. of Texas, Southwestern Medical School, Dallas, TX

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Growth hormone deficient subjects who meet the criteria for growth hormone therapy from the National Pituitary Agency are being selected for study. Serum lipoprotein composition is being measured before, during and after growth hormone therapy. Similarly, serum somatostatin and pancreatic polypeptide levels following a test meal are being measured in these same subjects to determine the effects of growth hormone therapy on these parameters.

PHS-6040

Project Description:

Previous clinical studies have indicated that growth hormone has many metabolic effects besides the stimulation of growth. Effects of growth hormone on carbohydrate, protein, and lipid metabolism have been demonstrated. Interactions with several hormones have also been noted.

Marked hyperlipidemia has been clearly demonstrated in growth hormone deficient adult dwarfs. Despite this hyperlipidemia and the abnormalities of carbohydrate metabolism found in adult dwarfs, the prevalence of arteriosclerosis is considerably lower than in diabetic subjects with similar abnormalities in carbohydrate and lipid metabolism. A recent study examined the effect of growth hormone therapy on serum triglycerides and cholesterol levels in growth hormone deficient children and found only minor changes in the levels of these lipids after treatment. Lipoprotein fractions were not separated in this study. Measurement of lipoprotein fractions in growth hormone deficient subjects before and during growth hormone therapy would be of interest because growth hormone deficient individuals might have altered lipoprotein distributions which might possibly protect growth hormone deficient dwarfs from vascular disease; moreover, alterations in their lipoprotein fractions may occur with growth hormone therapy.

Interrelationships between growth hormone and other hormones such as insulin have been well recognized. Somatostatin is known to suppress growth hormone secretion and a recent study in hypophysectomized dogs reported finding suppressed serum somatostatin levels suggesting a possible feedback relationship between these two hormones. Recent developments in the radioimmunoassay for somatostatin using ^{125}I-Tyrosine-11-somatostatin tracer to avoid problems of aminopeptidase degradation now make possible the determination of human serum somatostatin levels and therefore provide a methodology to determine if growth hormone deficiency or treatment are associated with altered somatostatin levels.

An association between growth hormone and pancreatic polypeptide has been suggested. One study has found pancreatic polypeptide secretion elevated in growth hormone deficient children. Furthermore, they found that growth hormone treatment did not suppress pancreatic polypeptide to normal levels in response to insulin hypoglycemia. Further examination of pancreatic polypeptide levels during growth hormone therapy might help to elucidate the relationship of these two hormones.

Objectives:

1. To examine the composition of serum lipoproteins before and during growth hormone therapy in growth hormone deficient subjects.

2. To examine the relationships between growth hormone and somatostatin and growth hormone and pancreatic polypeptide by measuring these hormones before, during and after growth hormone therapy in growth deficient subjects.

Methods Employed: Growth hormone deficient euthyroid subjects whose height is more than 2 standard deviations below the mean for age, have a current growth rate of <4 cm/yr, have a bone age delay of 2 or more years and an inadequate growth hormone response to 2 definite stimuli (insulin hypoglycemia, arginine infusion or L-Dopa) are considered for study. Each subject is admitted and placed on a weight-maintaining diet consisting of 45% CHO, 40% Fat and 15% Protein for 3 days. Then, after an overnight fast blood samples are obtained for plasma lipids and lipoprotein determinations and the measurement of somatostatin, pancreatic polypeptide, insulin, glucagon and glucose. The patient is then fed a test meal of 12-15 gm/kg composed of 25% Protein, 40% Fat and 35% CHO with blood sampling every 30 minutes for 3 hours for measurement of somatostatin, pancreatic polypeptide, insulin, glucagon, and glucose concentrations.

Major Findings: This study has commenced only recently and no results are available.

Significance to Biomedical Research and the Program of the Institute: This study should provide insight into some of the hormonal changes occurring in the growth hormone deficient state. Furthermore, it should provide better understanding of the interaction of growth hormone with somatostatin and pancreatic polypeptide. The study should also help elucidate the influence of growth hormone on plasma lipoprotein composition in humans.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54028-01 DD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Determination of Gastric Acid Secretion in Pima Indians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.L. Aronoff	Chief	PCRS, NIAMDD
Other:	M. Nagulesparan	Guest Worker	PCRS, NIAMDD
	A. Dubois		Dept. of Medicine Uniformed Health Sciences, School of Med., Bethesda, MD

COOPERATING UNITS (if any)
Eugene Strauss, V.A. Hospital, Bronx, NY
Roslyn Yallow, V.A. Hospital, Bronx, NY
I.L. McGregor, V.A. Med. Center, Tucson, AZ.

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Obesity and carbohydrate intolerance are common amongst the Pima Indians. It is possible that the development of obesity may in part be determined by the rates of gastric emptying. Furthermore, peptic ulcer disease is known to be low in the Pima Indian population, but increasing. We have also recently demonstrated that postprandial serum gastrin levels in diabetic Pima Indians are high. For the above reasons we plan to determine both gastric secretory and gastric emptying rates in Pima Indian subjects over a range of obesity, with and without diabetes.

Project Description:

Objectives: Obesity and carbohydrate intolerance are common among the Pima Indians. It is possible that the development of obesity may in part be determined by the rates of gastric emptying. The rapid emptying of food from the stomach would decrease the time of postprandial satiety consequent upon gastric distension by ingested food. Also, the more rapid transit of nutrients into the small intestine and the probably more rapid absorption would result in a peak of nutrient delivery to the body and encourage energy storage as neutral fat. An uncontrolled observation by radiologists is that barium sulfate suspension leaves the stomach of Pima Indians more rapidly than that observed in the Caucasian population. Furthermore, peptic ulcer disease is low in the Pima Indians population, but increasing. It is also known that postprandial serum gastrins in Pima Indians who are diabetic are high. For the above reasons, we plan to evaluate concurrently gastric emptying and gastric secretion of acid in Pima Indians over a range of obesity with and without diabetes. We expect to obtain answers to the following:

1. Do the fractional emptying rates correlate with the degree of obesity.
2. Are acid output and fractional emptying rates significantly different in the Pima Indians compared to a population with a greater frequency of peptic ulcer?
3. If the increase in the incidence of peptic ulcer disease among the Southwestern American Indian population continues, a subsequent study of gastric acid secretory rates may provide a better understanding of gastric acid secretion and peptic ulcer disease.
4. Does diabetes and glucose intolerance correlate with alterations in gastric emptying and gastric secretion?

Results: Pima Indian subjects were classified as normal (N), glucose intolerance (GI) or diabetic (D) on the basis of plasma glucose response to 100g oral glucose. Following a 12 hour fast plasma gastrin was measured by radioimmunoassay before and 15, 30, 60, 120, 180, 240 and 360 minutes after a test meal. Studies were carried out in 61 subjects (16D, 32N, 13GI). Fasting and postprandial gastrin concentrations were significantly elevated in the diabetic group. After the test meal diabetic subjects maintained serum gastrin levels in the range of 200 pg/ml for up to 2 hours and these levels were still almost twice that of normal and glucose intolerance subjects at 6 hours. It is concluded that among Pima Indian subjects diabetes is associated with fasting and postprandial hypergastrinemia.

Proposed Course: Gastric emptying for liquids and gastric secretion in both normal and diabetic Pima Indian subjects will be studied. Comparison will also be made with normal Caucasian subjects.

The rate of gastric emptying and the rate of secretion of acid and volume will be measured simultaneously using a dye dilution technique. A nasogastric tube will be passed into the stomach of a fasting subject. Serum glu-

cose, serum insulin and serum gastrin will be drawn at 15-minute intervals during the test. The test itself will consist of a 40-minute fasting period, a subsequent 60-minute period following intragastric administration of a 250 ml test water load (containing 40mg phenol red per liter, with or without 100 g glucose) and a final 60-minute period during which the normal saline infusion will be replaced by the intravenous infusion of a gastric secretagogue (pentagastrin; Peptavlon^{R}, Ayerst Lab, 6 g/kg/hr or histalog: Betazole^{R}, HCl, 40 g/kg/hr).

At ten minute intervals during the study, gastric contents will be aspirated and 5-20ml of a test solution identical in composition to the test meal will be injected into the stomach. At the end of the procedure, the stomach will be emptied completely. The concentration of phenol red and H^{+} ion will be determined in all samples. From these data, we will calculate the intragastric volume and acid content at each ten-minute time point, the amount of emptying in each ten-minute interval, and, by difference, infer the rates of secretion of acid and water.

Publications:

None

NIH Contract No: NO1-AM-6-2219 Starting Date: September 1, 1979
Expiration Date: August 31, 1980

Contractor: University of Texas Health Science Center
Southwestern Medical School

Money Allocated: $1,699,947.00 Man-years Purchased: 23.7

ANNUAL REPORT - 1979-1980

CLINICAL ENDOCRINOLOGY BRANCH

Dr. Weintraub received the Van Meter Armour Award of the American Thyroid Association, which was presented at the International Thyroid Congress in Sydney, Australia. This Award is given each year to a young scientist for outstanding contributions to thyroid research.

Two members of the Branch, Drs. Robbins and Wolff, participated as consultants in the Three Mile Island nuclear reactor accident which occurred in Pennsylvania. They arranged for monitoring of some of the potentially exposed population for evidence of radioactive iodine contamination, in cooperation with the Radiation Safety and Clinical Chemistry departments. No evidence of contamination was detected. They also provided advice on the use of potassium iodide for protection of the thyroid gland in the event of release of radioactive isotopes from the damaged reactor. Members of the Branch also served as consultants to the Food and Drug Administration (Drs. Robbins and Wolff), the National Council for Radiation Protection (Dr. Wolff), Brookhaven National Laboratory Marshall Islands Survey (Drs. Robbins and Wolff), and the National Pituitary Agency (Dr. Weintraub).

Visiting scientists from several foreign laboratories, including Switzerland, Finland, Italy and South Africa, participated in the research programs during the year.

I. Thyroid Biochemistry

A. Thyroid Cell Membrane and Mechanism of TSH Action

The solubilized adenylate cyclase from bovine thyroid cell membrane was shown in previous work to have a lower molecular weight (119,000) after fluoride activation than after GTP activation (159,000). Studies have been carried out to determine whether the missing 40,000 MW moeity might be the guanine nucleotide regulating protein. Since both stimulated forms of the adenylate cyclase are ADP-ribosylated under the influence of cholera toxin, it is suggested either that there are two GTP binding proteins in the catalytic complex, one of which is lost under the influence of fluoride, or that some other protein in the complex is subject to cholera toxin labeling. (Wolff, Goldhammer)

Previous studies showed that the thyroid cell has two types of surface binding sites for TSH. A high affinity site present in relatively small amounts has properties suggesting that it may be the physiologically active receptor. The most plentiful binding site has a much lower affinity for the hormone. On the

premise that the high affinity site may become disrupted during membrane purification, several methods were tested to see whether the yield of high affinity binding site could be enhanced. Isolation of cell membranes in high salt concentration (1 M ammonium sulfate at pH 5 and 37°) increased the yield of the high affinity site 4 to 8 fold. Prior treatment with trypsin prevented this increase, suggesting that the binding site is in an exposed position on the cell surface. The "new" binding sites obtained with high salt treatment were indistinguishable from those obtained without high salt, and there was no enrichment in the low affinity component after salt exposure. (Weintraub, Pekonen)

The removal of a considerable part of the cell membrane-bound carbohydrates, as reported previously, had no effect on TSH binding. On the other hand, it has now been shown that non-glycosylated TSH, obtained from cultured mouse pituitary tumor which was grown in the presence of tunicamycin, exists only as the free subunits. Furthermore, the coupling of these subunits to form the complete TSH molecule could not be achieved. Other studies with isolated fractions of tumor TSH which appear to differ in carbohydrate content showed differences in their receptor-binding and biological activity. These findings indicate that the oligosaccharides on the TSH molecule play a significant role in the activity of the hormone. (Weintraub)

B. Thyroid Hormone Secretion

The interaction of tubulin with the thyroid cell membrane is an important factor in thyroid hormone secretion. Previous work showed that iodination of tubulin results in damage which interferes with its ability to polymerize and self-iodinated lactoperoxidase surprisingly was found to form a complex with tubulin. This interaction has now been investigated in detail. It is a saturable and reversible reaction which leads to dissociation of the α and β subunits of tubulin and formation of a complex consisting of one molecule of lactoperoxidase with each of the subunits. The process is accelerated by diiodotyrosine, and involves one or more of the sulfhydryl groups on tubulin. Lactoperoxidase binds to preformed microtubules without displacing the microtubule-associated protein (MAP).

Other studies showed that tubulin has intrinsic high affinity and low affinity binding sites for Ca^{++} that vary with the state of polymerization. This reaction may be relevant to the role of calmodulin in the control of thyroid hormone secretion, and this aspect is now undergoing investigation. (Wolff, Rousset, Berkowitz)

C. Thyroid Hormone Synthesis

Previous work showed that, after intramolecular coupling of diiodotyrosines to form thyroxine in thyroglobulin, hydrolysis gives pyruvic acid as a product. It has now been proved that the "lost side chain" in the coupling reaction is dehydroalanine, which gives pyruvic acid during enzymatic hydrolysis. Several lines of evidence have been employed: addition of benzyl mercapton to give 5-benzyl cysteine residues, reduction with borohydride to give alanaine residues, and subsequent acid hydrolysis to give 5-benzyl cysteine or acetic acid, respectively. The stoichiometry indicated that one dehydroalanine residue was the only product for each thyroxine residue formed. Dehydroalanine is known to exist in bacterial antibiotic peptides, but in that case it is formed by a different synthetic mechanism. In thyroglobulin, dehydroalanine evidently is formed as a post-translational product during thyroid hormone synthesis. (Cahnmann, Gavaret, Pommier, Nunez)

D. Thyroxine-Protein Interactions

Thyroxine binding globulin (TBG), the major transport protein for thyroid hormone in the blood, is known to be easily denatured by heat and by slightly acid pH. In studies on the effects of guanadinium chloride, it was found that TBG is irreversibly denatured below 2 M GdmCl, resulting in randomization of half of the helical residues and loss of the hormone binding site. Above 2 M GdmCl, TBG undergoes several reversible molecular transitions. Thus, it is evident that TBG exists in plasma in a metastable state, indicating the probable occurrence of a post-translation modification of the protein. (Robbins, Edelhoch, Johnson, Gershengorn)

The thyroxine-binding site on TBG was investigated by affinity labeling with bromoacetyl thyroxine. Methionine was identified as the major residue labeled by this reagent, accounting for 75% of the products. The remainder was in lysine, tyrosine and histidine. This contrasts with previous findings with prealbumin, in which lysine was the major interacting residue. This is the first evidence that has been obtained thus far concerning the amino acid composition of the binding site on TBG. (Robbins, Cheng, Erard)

The level of free thyroid hormones in plasma is believed to govern the availability of thyroid hormones to the tissues. In order to aid in developing methods to test this hypothesis, a theoretical schema was developed which permitted the calculation of free or total hormone from the known concentrations and binding constants of the thyroxine transport proteins: TBG, prealbumin and albumin. The model was validated in various states

of abnormal TBG, and was used to simulate free T_4 and free T_3 in the "low T_3 syndrome" and in hypo- or hyperthyroidism. (Robbins, Johnson)

E. Thyroid Hormone-Cell Interactions

The use of video intensification fluorescence microscopy to study the entry of thyroid hormones into cells has been continued with cultured mouse fibroblasts. Rhodamine-labeled 3,5,3'-triiodothyronine ($RhoT_3$) became attached to the cell surface at a specific, saturable binding site. At 37°, these modular clustered and entered the cell by means of clathrin-coated, endocytic vesicles. The process was inhibited by methylamine, dansyl cadaverine and bacitracin. This is the first demonstration that a small molecule - in this case, a hormone - is taken into cells through the same mechanism as that operating with proteins such as insulin, nerve growth factor, and α_2-macroglobulin.

Whether this mechanism is an important means by which the thyroid hormone reaches its site of action is not yet clear. However, in studies with cultured monkey hepatoma cells, it has been shown that $RhoT_3$ incubated with intact cells is taken up by cell nuclei, and that this process is inhibited by other thyroid hormone analogs in a manner expected for the hormone receptor. (Cheng, Robbins, Pastan, Johnson)

F. Thyroid Hormone Metabolism

Alterations in thyroid hormone deiodinations are an important factor in the activation of thyroxine to form 3,5,3'-triiodothyronine, as well as in the degradation of the hormones. Several studies in other laboratories have suggested that the glutathione level in the cell may regulate the monodeiodination pathway. This hypothesis was investigated in continuously cultured monkey hepatoma cells and in primary cultured rat hepatocytes. Marked gluathione deficiency was produced by growing the cells in medium deficient in cystine and methionine, but this had no significant effect on deiodination. Oxidation of sulfhydryls by diamide, however, reduced both phenolic ring and nonphenolic ring deiodination. These findings show that reduced sulfhydryls are required for enzyme activation, but that intracellular glutathione levels do not regulate the enzyme activities. (Sato, Robbins)

Another detoxification reaction for thyroid hormones and their derivatives is sulfate conjugation at the phenolic hydroxyl group, a process which occurs primarily in the liver. The specificity of the sulfotransferase in monkey hepatoma cell extracts

was compared with that of two homogeneous sulfotransferases obtained from normal rat liver. By studying sulfate transfer to a series of hormone analogs, it was shown that derivatives containing one iodine atom in the phenolic ring are the best substrates, but that the three enzyme preparations exhibited a pattern of broad and overlapping specificity. (Cahnmann, Sato, Robbins, Sekura, Jakoby)

G. Thyroid Hormone Actions

The nuclear receptor for thyroid hormone in rat liver nuclei was studied by affinity labeling with N-bromoacetyl derivatives of T_3 and T_4. High performance liquid chromatography was used to achieve a 100-fold purification. An apparently identical receptor bound $BrAcT_3$, $BrAcT_4$, ^{125}I-T_3 and ^{125}I-T_4. Its molecular weight was about 56,000 and appeared to consist of a single polypeptide chain. (Nikodem, Cheng)

The study of thyroid hormone effects on individual proteins identifiable by 2-dimensional gel electrophoresis has been continued in rat liver nuclear extracts. The analytical technique was improved through the application of a computerized, photographic method. Using these procedures, a protein with a molecular weight of about 32,000 was found to be markedly diminished after thyroidectomy. Injection of T_3 into hypothyroid rats resulted in a gradual increment in this protein, reaching a maximum at 24 h and reverting to the hypothyroid level within 3 days. Studies have been begun on nonhistone chromosomal proteins having high electrophoretic mobility (so-called HMG proteins) which appear to be involved in DNA translation. Acetylation of these proteins was increased in HMG preparations of hypothyroid rats compared to control. (Nikodem, Rall)

H. Studies of Thyroid Disease

Previous work showed that a suitable regimen for radioiodine testing for thyroid cancer metastases is to replace T_4 with T_3 for 4 weeks and then stop all therapy for 2 additional weeks. Blood obtained from patients in this study are now being tested by thyroglobulin radioimmunoassay. Preliminary evidence indicates that a state of hypothyroidism is needed to stimulate thyroglobulin appearance in bood, and that thyroglobulin can be a sensitive indicator of residual thyroid tissue. The relative value of thyroglobulin measurement compared to ^{131}I scanning is now being evaluated. (Robbins, Weintraub, Goldman, Schneider, Pretorius, Fradkin)

Studies have now been carried out on the two available patients with the syndrome of inappropriate TSH secretion, in

order to examine the rates of metabolism of thyroxine and its deiodinated derivatives. Both patients had lower than normal metabolic clearance rates (MCR) of T_4, but normal MCR of T_3 and reverse T_3. The production rate (PR) of T_4 was normal in one patient and low in the other. The PR of T_3 and rT_3 were high compared to normal, but considerably lower than in hyperthyroid patients with similar T_4 levels or in some other patients with inappropriate TSH syndrome. At least one form of this syndrome may result from abnormal cellular uptake of the hormone, and this possibility is now under investigation in cultured fibroblasts. (Weintraub, Fein)

Several additional patients with the new syndrome of postpartum transient hypothyroidism have been identified. This syndrome is found in women with lymphocytic thyroiditis, and prospective observation of individual subjects shows the occurrence first of transient hyperthyroidism, followed by transient hypothyroidism. Since symptoms of mild hyper- and hypothyroidism in the first year postpartum may be easily overlooked, the incidence of this disorder is unknown. This is currently under investigation by screening a large group of women folloiwng delivery. (Weintraub, Fein)

The effect of lithium as a supplementary treatment after low dose ^{131}I therapy of hyperthyroidism is being compared with the effects of iodide. Both agents control the hyperthyroid syndrome, but iodide is much more likely to produce hypothyroidism, and it does so at a relatively much lower dose. Lithium is more toxic, but may be useful in treating thyrotoxic "storm", (Weintraub, Robbins, Fradkin)

A randomized study of the treatment of Graves' ophthalmopathy employing lymphopheresis and plasmapheresis has been initiated. Up to this time, objective evidence of a change in eye signs has been minimal, but a dramatic decrease in thyroid-binding immunoglobulins (TBI) has been observed in some patients. In these initial studies, patients with relatively stable ophthalmopathy have been selected; it is possible that those with more acute changes may be more likely to experience a remission. (Weintraub, Fein, Ballantine)

II. A. Biosynthesis of Glycoprotein Hormones

Further progress has been made on the amino acid sequence of the "pre"-α subunit obtained by cell free synthesis in a wheat germ system. In comparing normal bovine pituitary, normal mouse pituitary and mouse thyrotropic tumor, as well as human placental pre-α, the structure of which was recently published by others, it was found that at least 8 amino acids have been strictly conserved in the hydrophobic "signal" peptide.

This structure apparently plays a vital role in the passage of newly synthesized hormone into the endoplasmic reticulum. (Weintraub, Giudice)

Using mouse thyrotropic tumor cells and tunicamycin, an antibiotic which prevents glycosylation of glycoproteins, a new 12,000 dalton αsubunit has been obtained. Although this carbohydrate-free hormone can be secreted by the cell, it is unable to combine with the β unit to form intact TSH. (Weintraub)

Continued study of the carbohydrates of human chorionic gonadotropin has shown that intracellular free α, extracellular free α, and α obtained by dissociating intact hCG, all differ in their carbohydrate structure. Furthermore, as in the case of TSH, polymerization of hCG can occur only from the glycosylated subunits. (Weintraub, Rosen, Dean)

B. Ectopic Polypeptide Hormone Syndromes

SP_1 is a β_1-glycoprotein found in pregnancy. Because this protein was observed as a product of human fibroblasts in culture, but is not present in normal plasma, the possibility that it might be associated with fibroblastic tumors was investigated. However, no elevations were found in benign or malignant tumors of this type (neurofibromatosis, fibrosarcoma, glioma, etc.). It also was not found in significant quantity in breast carcinoma, despite other reports to the contrary.

The nutritional requirement of HeLa cells for producing hcG-α and hCG was investigated. Conditions were found which permit excellent hCG formation in the absence of fetal calf serum, thus simplifying the study of ectopic hCG-α biosynthesis in these cells. (Rosen, Weintraub, Morrow)

C. Gonadotropin Pathophysiology

The mode of inheritance of Kallmann's Syndrome and associated phenotypic abnormalities (e.g. cleft lip or palate, cryptorchidism) was examined in 18 patients and their families. The studies suggest that there is genetic heterogeneity in this syndrome. Both X-linked transmission and autosomal inheritance appear to occur. (Rosen, Morrow)

III. Steroid Hormone Secretion

Further studies have been carried out on the interaction of adenosine with adrenal cortical cell and other membranes. Inhibitory and stimulatory receptors can be discriminated by selected adenosine analogs. 5'-N-ethyl carboxamide-adenosine is more potent than N^6-phenylisopropyl-adenosine when tested on the stimulatory receptor, whereas the reverse order is seen with the inhibitory

receptor. Adenosine itself has intermediate potency. The relative potency of the analogs is maintained in whole cell systems, as tested by steroidogenesis and inhibition of lipolysis. It appears that distinct subclasses of adenosine receptors exist, as is also seen with adrenergic receptors. (Wolff, Londos)

IV. Studies on Protein Structure and the Interaction of Proteins with Cell Membranes

In previous studies, the molecular properties of clathrin isolated from bovine brains were defined. Techniques have now been devised to study the kinetics of polymerization of clathrin into so-called "baskets", which appears to be a model for the formation of endocytic vesicles. These techniques utilize absorbance and light scattering measurements. It has been found that certain basic proteins, such as lysozyme, profoundly enhance the rate of polymerization. Small molecules such as reserpine appear to bind to unpolymerized clathrin and then increase its rate of polymerization. The relationship of these in vitro phenomena to the cellular process of endocytosis is under investigation. (Edelhoch, Nandi, van Jaarsveld, Pretorius)

V. Mechanism of Hormone Action. Adenylate Cyclase in Bacteria

Continuation of studies on the adenylate cyclase of Bordetella pertussis has revealed that it is stimulated up to 1000 fold by a protein activator. Since this activator resembles calmodulin in its heat stability, the effect of calf brain calmodulin on the bacterial adenyl cyclase was investigated. It was shown that the cyclase is stimulated by calmodulin in a manner identical to that observed with eukaryotic adenyl cyclsae. These experiments gave the first demonstation of an effect of calmodulin in a prokaryote, and suggest that a calmodulin-like system exists within this organism. (Wolff, Goldhammer, Berkowitz)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45000-13 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Thyroxine Protein Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	F. Erard	Visiting Fellow	CEB NIAMDD
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COOPERATING UNITS (if any)
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SECTION
Endocrine Physiology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.0	0.6

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Thyroxine binding globulin (TBG) has been studied from several points of view. Physical properties of the molecule were investigated as affected by guanadinium chloride. The results indicated that the native molecule in the serum is in a metastable state since mild denaturation conditions lead to irreversible changes in molecular properties and loss of the binding site for thyroid hormone. Affinity labeling of TBG with bromacetyl thyroxine showed that the major amino acid interacting with this probe in the binding site was methionine. Minor amounts of lysine, tyrosine and histidine were also labeled.

A theoretical schema for the calculation of the free thyroid hormones was developed utilizing known parameters of hormone binding in serum.

PHS-6040

Project Description:

Thyroxine binding globulin (TBG) is a specific transport protein for the thyroid hormones in human plasma. It is a glycoprotein with a molecular weight of 54,000 and a single polypeptide chain. The molecular transitions of TBG in guanidinium chloride solutions were evaluated by circular dichroism, tryptophanyl fluorescence and polarization of tryptophanyl fluorescence at neutral pH. Below 2 M guanidinium chloride, the protein underwent a time dependent, irreversible transition which involved a randomization of almost half of the α-helical residues. The hormone binding site was irreversibly lost during this transition. At higher guanidinium chloride concentrations (2-5 M), TBG underwent several further reversible molecular transitions. These data indicate that the native TBG molecule as it exists in serum is not the most stable form of the protein. Our previous work had shown a similar metastability of TBG when exposed to dilute acid. The findings raise questions about possible transitions in TBG following its synthesis and/or secretion from the hepatocyte.

The thyroxine binding site in TBG was investigated by affinity labeling with n-bromo-acetyl-L-thyroxine ($BrAcT_4$). Competitive binding studies showed that, in the presence of one hundred molar excess of $BrAcT_4$, binding of thyroxine to TBG was nearly totally abolished and that the reaction of $BrAcT_4$ to form covalent binding was inhibited in the presence of thyroxine. Furthermore, affinity labeled TBG lost its ability to bind thyroxine. These results indicate that $BrAcT_4$ and thyroxine compete for the same binding site. Amino acid and analysis and high-voltage paper electrophoresis analysis of the affinity labeled TBG identified methionine as the major residue labeled by the bromo-acetyl derivative, accounting for 75% of the covalent labeling. Lysine, tyrosine and histidine were also labeled to the extent of 8, 8, and 5%, respectively. This result is in contrast to that previously found with prealbumin, the other major thyroxine transport protein in serum. In the case of PA, most of the bromo-acetyl thyroxine labeling was with lysine. In the absence of availablity of crystals of TBG suitable for X-ray defraction study, it is hoped that the method of affinity labeling will help to clarify the nature of the binding site on this protein.

Most of the available evidence indicates that the levels of free thyroid hormones in plasma are responsible for the maintenance of normal thyroid status in any individual. We proposed and developed a theoretical schema which explores the interaction of the hormones and the proteins in blood. The mathematical formulation was derived from statistical thermodynamics, and the solution was obtained by the Marquardt-Levenberg algorithm utilizing the MLAB modeling program. Literature values for normal serum levels of thyroxine, triiodothyronine, TBG, PA and albumin, and the

association constants at nearly physiological conditions, were used to calculate normal free T_4 and free T_3 levels in serum and cerebrospinal fluid. The close agreement between the calculated and measured values indicate that the methods and the constants were reasonable. We then used these methods to evaluate the effects of temperature on free hormone levels, to simulate the changes expected in various forms of hyperthyroidism, and the "low T_3 syndrome", and to calculate total T_4 and T_3 levels in cases of genetically altered transport proteins. It is proposed that this technique could be used to test for the possible presence of abnormal transport proteins or other factors affecting the interactions and to evaluate the validity of the equilibrium hypothesis for the free thyroid hormones in abnormal states.

Publications:

1. Robbins, R., Johnson, M.: Theoretical Considerations in the Transport of the Thyroid Hormones. In Ekins, R., Faglia, G., Pennisi, F., and Pinchera, A. (Eds.): Free Thyroid Hormones. Amsterdam, Excerpta Medica, 1979, pp. 1-14.

2. Johnson, M., Lippoldt, Gershengorn, M., Robbins, J. and Edelhoch, H.: Molecular transitions of human thyroxine binding globulin. Archives Biochem. Biophys. 200: 288-295, 1980.

3. Cheng, S.-Y., Morrone, S. and Robbins, J.: Effect of deglycosylation on the binding and immunoreactivity of human-thyroxine binding globulin. J. Biol. Chem. 254: 8830-8835, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45003-06 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Model Reactions for the Synthesis of Thyroxine Residues in Thyroglobulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
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Endocrine Biochemistry

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.6	.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

For each thyroid hormone residue formed when iodine-free newly synthesized thyroglobulin is iodinated enzymatically (thyroid peroxidase), one dehydroalanine residue is formed. Thus, protein-bound dehydroalanine is a side product of thyroid hormone synthesis in thyroglobulin.

Project No. Z01 AM 45003-06

Project Description:

We reported last year (Z01 AM 45026-01 CEB) that the enzymatic hydrolysate of [U-^{14}C]tyrosine labeled hog thyroglobulin contains [^{14}C]pyruvic acid. We have now determined that this pyruvic acid is derived from dehydroalanine residues. Iodine-free, newly synthesized thyroglobulin contains neither hormone (T_4+T_3) nor dehydroalanine residues. Both types of residues are formed concomitantly upon enzymatic iodination in a molar ratio of 1.

Dehydroalanine residues have been quantitated by two independent methods: (1) addition of benzyl mercaptan leading to the formation of S-benzylcysteine residues, and (2) reduction with $NaBH_4$ leading to the formation of alanine residues. Acid hydrolysis (HCl), following reactions (1) and (2), yielded free S-benzylcysteine (method 1) or [^{14}C]acetic acid (method 2). In the latter case, treating with HCl converts the originally formed pyruvic acid to acetic acid. Protein-bound dehydroalanine is the only product of the "lost side chain" which remains when two iodothyronine residues in thyroglobulin undergo a "coupling reaction" to form a hormone residue. The biogenesis of dehydroalanine residues in thyroglobulin is different from that of dehydroalanine residues formed in many bacterial antibiotic peptides which seem to be devoid of serine or cysteine residues.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45004-09 CEB

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure of Polypeptide and Protein Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	H. T. Pretorius	Staff Fellow	CEB NIAMDD
	P. Nandi	Visiting Scientist	CEB NIAMDD
	P. van Jaarsveld	Visiting Scientist	CEB NIAMDD
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COOPERATING UNITS (if any)

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LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Protein Structure

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.8	3.7	1.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are continuing our study of receptor mediated endocytosis by the coated pit regions of cellular membranes. We have isolated coated vesicles from bovine brains and extracted the protein responsible for the coat. It is supposed tha the protein coat may control the size of the vesicle and regulate the formatio of coated vesicles from coated pits during endocytosis.

PHS-6040

Project Description:

We have polymerized purified preparations of clathrin into baskets. These baskets have the same or similar structural parameters as the coat of coated vesicles. The distribution of basket sizes have been analyzed by sedimentation velocity and the molecular weights by sedimentation equilibrium and light scattering.

A kinetic assay of clathrin polymerization to baskets has been developed by absorbance and light scattering techniques. This permitted the determination of the effects of clathrin concentration, pH, ionic strength and temperature on the rate of polymerization.

It was found that certain basic proteins, e.g. lysozyme, can enhance the rate of clathrin polymerization many-fold. In fact, the rate could be measured at physiological pH, i.e. 7.5, whereas in the absence of lysozyme, the pH has to be reduced below ~ 6.5 for polymerization to occur at a measurable rate.

We have also begun to screen some small molecules where there is reason to believe that they may be involved in endocytosis. We have found that reserpine is bound to unpolymerized clathrin and increases the rate of clathrin polymerization. In this way, various regulators may be able to influence the rate of endocytosis of hormones and other proteins by controlling coated vesicle formation from coated pits.

Publications:

1. Edelhoch, H., Irace, G., Johnson, M. L., Michot, J. L., and Nunez, J.: The effects of thioureylene compounds (goitrogens) on lactoperoxidase activity. J. Biol. Chem. 254: 11822-11830, 1979.

2. Johnson, M. L., Lippoldt, R. E., Gershengorn, M. C., Robbins, J., and Edelhoch, H.: Molecular transitions of human thyroxine-binding globulin. Arch. Biochem. Biophys. 200: 288-295, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-45009-13 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies in Thyroid Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.5	.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In the diagnosis of thyroid nodules and goiter, further data has been accumulated on the utility of fine needle aspiration cytology in preoperative diagnosis. This has also been an effective means of diagnosing lymphocytic thyroiditis. In the treatment of thyroid cancer, the serum thyroglobulin levels are being evaluated in correlation with the previously completed study of whol body ^{131}I scanning. The method promises to be useful provided that patients are allowed to become at least mildly hypothyroid. In the treatment of hyperthyroidism, it was found that both iodide and lithium are effective in controlling hyperthyroidism after the use of relatively low dose ^{131}I therapy. In the amounts used, iodide produced a greater response than lithium. In the syndrome of inappropriate secretion of thyrotropin (TSH), iodothyronine metabolism was studied in two patients. The metabolic clearance of T_4 and T_3 were low relative to their circulating hormone levels, but the conversion of T_4 to T_3 was not impaired. This suggests a possible defect in the entry of thyroid hormones into cells. In the syndrome of postpartum thyroiditis with transient hypothyroidism, several additional patients have been detected and studied.

Project Description:

1. Diagnosis of Thyroid Nodules and Goiter

Investigation of the utility of fine needle aspiration biopsy is continuing with accumulation of more cases of thyroid nodule and various types of goiter. We find that it is possible in some cases to make an unequivocal diagnosis of papillary carcinoma of the thyroid and of lymphocytic thyroiditis. In the first case, this leads to immediate surgery avoiding the usual delay in attempts at suppression therapy which is customary in management of thyroid nodules. In the second instance lymphocytic thyroiditis can be identified as part of a study of the natural history of this disease, particularly in relation to postpartum hypothyroidism. Also it can avoid the necessity for surgical biopsy in some cases. In collaboration with the Department of Radiology the use of thyroid echography as an aid in the placement of the aspiration needle into the diseased area of the thyroid, either solid or cystic, is being evaluated.

2. Treatment of Thyroid Cancer

The third phase of our study on methods for diagnosis of thyroid cancer metastases is in progress. Serum collected from patients in whom radioiodine testing had been done at various times after triiodothyronine withdrawal are now being studied by radioimmunoassay of thyroglobulin. Initial results indicate that this is a valuable method to detect the presence of functioning thyroid tissue after the thyroid gland has been ablated by surgical thyroidectomy or ablation with radioactive iodine. However, some degree of hypothyroidism is required for serum thyroglobulin to become elevated. We are continuing to evaluate the adequacy of radiation thyroidectomy with relatively small doses of radioactive iodine; i.e., 30 mCi of ^{131}I. This dose can be administered to outpatients, and complete thyroidectomy can usually be accomplished with from one to three doses given at intervals of six months. In the radioiodine therapy of metastatic thyroid cancer, additional patients have been studied to evaluate the utility of lithium as a means of slowing the release of ^{131}I from the tumor and thus improving the tumor/whole body radiation exposure.

3. Treatment of Hyperthyroidism

Patients receiving ^{131}I therapy for hyperthyroidism are being studied to evaluate the post-therapy use of potassium iodide and lithium to control the hyperthyroidism following the use of relatively small doses of ^{131}I. The intent is to use these small doses in order to decrease the late development of hypothyroidism, which is known to occur following such therapy. We have found that potassium iodide adequately suppresses thyroid function in the radiated gland so that the patients can be maintained for long periods of time in a euthyroid state. In the same patients, we are comparing

the effects of lithium with those of potassium iodide. Preliminary results indicate that lithium is also an effective suppressor of thyroid hormone secretion. In the doses used, lithium is less effective than potassium iodide, and is less likely to produce symptomatic hypothyroidism. In a later phase of this study, the combined use of lithium and iodide will be employed to determine whether the effects of these two agents are additive or synergistic. This is part of an attempt to evaluate the usefulness of lithium in the treatment of thyrotoxic storm.

4. Inappropriate Secretion of TSH

We have studied iodothyronine metabolism in 2 patients with the syndrome of inappropriate secretion of TSH. The metabolic clearance rates for T_4 were below euthyroid controls for both patients, while clearance rates for T_3 and reverse T_3 were within the control range. The production rate for T_4 was far below control in one patient who had T_3 and rT_3 production within control range, resulting in elevated T_4 to T_3 and T_4 to rT_3 fractional conversion rates. The other patient had T_4 production within the control range, with moderately elevated production of T_3 and reverse T_3. In both patients, these iodothyronine clearance and production rates were far below those reported for conventionally hyperthyroid patients and for certain other patients with inappropriate TSH secretion. This implies that there may be heterogeneous biochemical mechanisms involved in the pathogenesis of this disorder. At least one form of the syndrome may result from abnormal cellular uptake of thyroid hormones. Preliminary studies of T_3 binding to nuclear receptors in circulating lymphocytes showed normal or slightly elevated binding in the one patient studied. Skin fibroblasts are being cultured from 3 patients and studies have been initiated to examine nuclear and cell membrane binding of thyroid hormones as well as thyroid hormone uptake and action in these cells.

5. Postpartum Thyroiditis with Transient Hypothyroidism

We have previously identified five patients with a new syndrome of autoimmune thyroiditis with transient hypothyroidism, previously only described in a single Japanese series. We have recently identified several new patients with this syndrome and have begun a prospective study of pregnant women with a goiter and/or history of Hashimoto's thyroiditis. Three women have given birth and are being evaluated at monthly intervals in order to more fully document the clinical course of this disorder. We are also beginning to study a number of *in vitro* measurements of lymphocyte function in these women during and after pregnancy and hope to define the immunologic basis of this disorder.

Publications:

1. Goldman, J., Line, B., Aamodt, R., and Robbins, J.: Influence of triiodothyronine withdrawal time on ^{131}I uptake post-thyroidectomy for thyroid cancer. J. Clin. Endo. Metab. 50: 734-739, 1980.

2. Robbins, J., Rall, J. E., and Gorden, P.: In Bondy, P. K., and Rosenberg, L. E. (Eds.): Metabolic Control and Disease, 8th Edition. W. B. Saunders, Philadelphia, 1980, pp. 1325-1425.

3. Robbins, J. and Rall, J. E.: In Gray, C. H., and James, V.H. T. (Eds.): Hormones in Blood, 3rd Edition, Vol. 1. Academic Press, London, 1979, pp. 576-688.

4. Chu, E. W., Hansen, T. A., Goldman, J., and Robbins, J.: Study of cells in fine needle aspirations of the thyroid gland. Acta Cytologica J. 23: 309-314, 1979.

5. Gershengorn, M. C., Glinoer, D., and Robbins, J.: In De Visscher, M. (Ed.): The Thyroid Gland. Raven Press, New York, 1980, pp. 81-121.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45010-10 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Ectopic Protein and Subunit Production by Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Rosen	Medical Director	CEB NIAMDD
	B. D. Weintraub	Medical Director	CEB NIAMDD
	J. S. Morrow	Clinical Associate	CEB NIAMDD
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	J. Osborne	Sr. Investigator	IR MD NHLBI
	E. Heyderman	Sr. Lecturer, Dept. Path.	St. Thomas's Hosp. Med. Sch., London
	D. Tormey	Assoc. Prof., Dept. Med.	U. Wisc. Med. Ctr.
	C. Horne	Prof., Dept. Path.	U. of Aberdeen, Scotland
	D. Zopf	Sr. Investigator	LP NCI

COOPERATING UNITS (if any)

National Cancer Institute; St. Thomas's Hosp. Med. Sch., London, England; University of Aberdeen, Scotland; Univ. of Wisconsin Med. Ctr.

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.3	.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We continue to study placental proteins as cancer markers. The pregnancy specific beta$_1$ glycoprotein (SP$_1$) is produced by normal human fibroblasts but does not appear to be a useful marker in cases of fibrosarcoma, osteogenic sarcoma, glioma or breast carcinoma. Placental protein 5 is also made by human fibroblasts *in vitro* but its regulation by the cell appears to be differen from that of SP$_1$. Chemical modification of SP$_1$ is being explored in an attemp to understand its unusual CD spectrum. Placental lactogen (PL) is made by dif ferent cells of a testicular choriocarcinoma than is alpha subunit. Large forms of PL have been detected in serum and placenta at term, even when blood and tissue have been rapidly processed. Analysis of oligosaccharides released from CG-α by trifluoroacetolysis has revealed heterogeneity unrecognized in current structural formulas of this glycoprotein. HeLa cells make α subunit in serum-free media with growth factor supplementation, at rates up to 80% of those in standard medium containing fetal calf serum.

Project Description:

A. In Vivo Studies

The biologic role of SP_1, a recently identified $beta_1$-glycoprotein of pregnancy, has not yet been determined. We and others have found it in serum from women and men with trophoblastic neoplasia. We also found this glycoprotein in media and cells of virtually every human fibroblast line tested. In one fibrosarcoma line, its production rate was increased 100-fold over normal fibroblasts. Its concentration in normal human serum, however, is very low (< 4 ng/ml) if, indeed, it is present at all.

It occurred to us that the concentration of SP_1 might be increased in situations of fibroblastic proliferation. Accordingly, we examined sera from patients with benign (neurofibromatosis, primary pulmonary hypertension) and malignant (fibrosarcomas, other sarcomas, gliomas) lesions of fibroblasts or related cell types. No patients with benign tumors, gliomas or osteosarcomas had SP_1 elevation. Two of 32 patients with soft tissue sarcomas had elevated SP_1. The level in one man (133 ng/ml) was even higher than that in most patients with nonseminomatous germ cell cancer of the testis. This observation is being pursued.

In one study from Europe, SP_1 was touted as a marker for breast carcinoma. We examined blind coded serum samples from a Midwest cooperative breast cancer study. The serum SP_1 from 71 cancer patients was rarely over one standard deviation above 61 normals and 33 women with benign breast disease analyzed in the same assay. The cancer group included women with all stages of this disease. We conclude that SP_1 is not a useful marker in carcinoma of the breast.

B. In Vitro Studies

The method of immunocytochemistry shows great promise in identifying the cell(s) of origin of eutopic and ectopic placental proteins. We have used this technique to examine a metastatic testis choriocarcinoma and have found that different cells make placental lactogen and alpha subunit. We are now re-examining a gastric carcinoma that produced CG-β and placental lactogen (PL) to see whether PL is made by the large trophoblast-like giant cells in which we earlier demonstrated CG-β production.

HeLa cells have been shown to ectopically produce hCG-α subunit and/or intact hCG. In addition, the growth of these cells can be maintained for long periods of time in a serum-free medium supplemented with insulin, hydrocortisone, transferrin, EGF, and FGF. Very little is known about the ability of this medium to support cellular functions other than growth, and for that reason we investigated hCG-α production by HeLa-S_3 cells grown in the above medium. Our studies show that, depending on the

growth factors added, hCG-α secretion into the serum-free media can be maintained at 50-80% of the level at which it occurs in media supplemented with 10% fetal calf serum. This finding will enable us to study further the in vitro biosynthesis of ectopic hCG-α in the absence of the many unknown factors present in serum which could conceivably affect this process.

We have isolated messenger RNA from term human placentas as well as from HeLa cells grown in athymic ("nude") mice. We are attempting to translate these messages in a cell-free system using wheat germ extract. After first characterizing the resulting hCG-α subunits by physiochemical means and comparing them with hCG-α subunit dissociated from urinary standard hCG, we will then study regulation of ectopic hCG-α biosynthesis.

Study of SP_1 in the equilibrium ultracentrifuge has revealed that this glycoprotein is a homodimer with a subunit molecular weight (in 7 M guanidine) of 44,700. Electrophoretic analysis on gradient polyacrylamide gels in the presence of SDS has shown a dominant band (> 90%) with apparent mol wt of 73,000 - the discrepancy presumably reflecting the usual decreased binding of SDS by glycoproteins. SP_1 has an interesting CD spectrum, with a small positive peak at 235 nm and an unusually shallow (2600) negative peak at 215 nm. The positive ellipticity of the homodimer was abolished in a solution of 3.5 M guanidine-1 mM cystine, conditions which did not lead to dissociation. We are currently examining the effects of serial carbohydrate removal on the spectral properties of SP_1.

Chromatography of third trimester sera on dextran and on polyacrylamide gels revealed a large form of PL eluting near the void volume of the column. This large form has been seen even when serum taken from the patient was applied to the column within 60 min of collection. A similar form has been found in gel chromatography of freshly extracted placenta from the same patients. Studies are in progress to characterize this material.

PP_5, a newly described placental glycoprotein, has been found in media from most cultured normal human fibroblasts examined, much as we showed earlier for SP_1 - but unlike CG-β and PL, which were rarely, if ever, detected in assays of comparable sensitivity. PP_5 and SP_1 production by the same fibroblast line was discordant, implying differences in their cellular regulation.

Sugar analysis of whole CG-α and of the oligosaccharides released by trifluoroacetolysis of CG-α gave monosacacharide ratios incompatible with the published structure (Bahl et al.) for asparagine-linked carbohydrate in this glycoprotein. Methylation analysis suggests that these chains are heterogeneous and that some contain at least one non-reducing N-acetylglucosamine residue. Further chemical studies are in progress

to elucidate the detailed carbohydrate structure of CG-α; these micromethods will ultimately be applied to the much smaller quantitities available for analysis of the alpha subunits of LH, FSH and TSH.

Publications:

1. Dean, D. J., Weintraub, B. D., and Rosen, S. W.: De novo synthesis and secretion of heterogeneous forms of human chorionic gonadotropin and its free alpha subunit in the human choriocarcinoma clonal cell line, JEG-3. Endocrinology 106: 849-858, 1980.

2. Neuwald, P. D., Anderson, C., Salivar, W. O., Aldenderfer, P. H., Dermody, W. C., Weintraub, B. D., Rosen, S. W., Nelson-Rees, W. A., and Ruddon, R. W.: Expression of oncodevelopmental gene products by human tumor cells in culture. J. Natl. Cancer Inst. 64: 447-459, 1980.

3. Rosen, S. W.: Paraneoplastic syndromes: Something old, something new. West. J. Med. 132: 240-241, 1980.

4. Rosen, S. W., Weintraub, B. D., and Aaronson, S. A.: Nonrandom ectopic protein production by malignant cells: Direct evidence in vitro. J. Clin. Endocrinol. Metab. 50: 834-841, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45013-08 CEB

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biosynthesis of Glycoprotein Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. D. Weintraub	Medical Director	CEB NIAMDD
	S. W. Rosen	Medical Director	CEB NIAMDD
OTHER:	B. S. Stannard	Physicist	CEB NIAMDD
	H. Fein	Clinical Associate	CEB NIAMDD
	D. Dean	Staff Fellow	CEB NIAMDD

COOPERATING UNITS (if any)

NIAID

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Physiology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	1.9	.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Microsequencing of the signal peptides from pre-α subunits synthesized in cell-free systems reveals the strict conservation of at least 8 hydrophobic amino acids among 3 different species and 4 different tissues. Tunicamycin causes the formation of nonglycosylated α subunits which can be secreted but cannot combine with β subunits. Free and combined α subunits appear to have a different carbohydrate structure. Deglycosylation of HCG-α with a mixture of exoglycosidoses prevents its recombination with intact glycosylated HCG-β.

PHS-6040

Project Description:

I. Biosynthesis of TSH

A. Cell-free translation of messenger RNA

We have continued microsequencing studies of pre-α subunits synthesized in a cell-free wheat germ system. Several additional amino acid positions have been elucidated in the α signal peptide synthesized from 3 different messenger RNA's: normal bovine pituitary, normal mouse pituitary and mouse thyrotropic tumor. These structures have been compared to the full structure of human placental pre-α which has been recently described by Fiddes and and Goodman (Nature 281: 351, 1970). Figure 1 (attached) shows that there are at least 8 amino acids that have been strictly conserved in the pre-α's derived from 3 different species and 4 different tissues. Besides the initiating methionine, there are 3 identical tyrosines as well as 4 identical leucines. These hydrophobic amino acids appear to be vital for translocation of the nascent pre-α into the rough endoplasmic reticulum as well as the cleavage of the signal peptide. In contrast, recent studies indicate different amino acids among species at positions 24-25, the presumed site of cleavage.

B. Studies in dispersed thyrotroph cells

We have continued studies of de novo TSH biosynthesis in intact mouse thyrotropic tumor cells. The addition of tunicamycin, which inhibits formation of the lipid-oligosaccharide intermediate necessary for glycosylation at asparagine residues, causes the appearance of a new 12,000 dalton α form which incorporates [^{35}S]-methionine but not [^{3}H]-glucosamine. This nonglycosylated α does not combine with β but can be secreted.. This form also appears to be rapidly degraded by intracellular proteases in contrast to normally glycosylated α.

We are currently performing very early (1-10 min) pulse-chase studies of the incorporation of various labeled amino acids and carbohydrates into TSH. Recent data suggest that little pre-α or pre-β accumulates in the intact cell; rather, the signal peptide is probably cleaved before completion of the nascent chains. Early processing events are confined to modification of the high-mannose core sugars. Such modifications appear necessary before α-β subunit combination can occur.

Pre-α	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Mouse Tumor	Met	X	Tyr	Tyr	X	X	Tyr	X	X	X	X	Leu	(Cys)	Met	Leu	X	Met	X	Leu	X	X	Leu	X
Mouse Pituitary	Met	X	X	X	X	X	X	X	X	X	X	X	X	Met	X	X	Met	X	X	X	X	X	X
Bovine Pituitary	Met	X	X	X	X	X	X	X	X	X	X	Leu	X	X	Leu	X	Leu	X	Leu	X	X	Leu	X
Human Placenta	Met	Asp	Tyr	Tyr	Arg	Lys	Tyr	Ala	Ala	Ile	Phe	Leu	Val	Thr	Leu	Ser	Val	Phe	Leu	His	Val	Leu	His

II. Biosynthesis of HCG

A. Choriocarcinoma cell lines

We are continuing studies of HCG synthesized by the choriocarcinoma cell line, JEG-3. Recent studies suggest that there is a different carbohydrate structure of the free extracellular α compared to extracellular α combined with β as well as compared to free intracellular α. We plan to characterize these differences by preparing labeled glycopeptides from each source and analyzing them by gel and thin-layer chromatography.

Tunicamycin treatment also produces a 12,000 dalton non-glycosylated α which does not combine with β but can be secreted. However, since the β subunit contains serine-linked carbohydrates, tunicamycin results in only partially deglycosylated β of intermediate molecular weight.

B. Role of carbohydrate in combination of HCG subunits

Using a protease-free mixture of several exoglycosidases, we have partially deglycosylated the α subunit dissociated from urinary hCG and tested its ability to recombine with intact glycosylated HCG-β. By both immunoassay and receptorassay techniques, there was little or nor recombination of deglycosylated α with β, while normal α displayed 100% recombination. We plan to evaluate individual glycosidase enzymes for their effects on both α and β combining properties. These studies provide direct in vitro evidence for the importance of carbohydrate in subunit combination.

Publications

1. Condliffe, P. G., and Weintraub, B. D.: Pituitary Thyroid-stimulating Hormone and Other Thyroid-stimulating Substances. In Gray, C. H., and James, V. H. T. (Eds.): Hormones in Blood, 3rd Edition. London, Academic Press, 1979, pp. 499-574.

2. Giudice, L. C., Waxdal, M. J., and Weintraub, B. D.: Comparison of bovine and mouse pituitary glycoprotein hormone pre α subunits synthesized in vitro. Proc. Nat. Acad. Sci. (USA) 76: 4798-4802, 1979.

3. Giudice, L. C. and Weintraub, B. D.: Evidence for conformational differences between precursor and processed forms of thyroid-stimulating hormone β subunit. J. Biol. Chem. 254: 12679-12683, 1979.

4. Dean, D. J., Weintraub, B. D. and Rosen, S. W.: De novo synthesis and secretion of heterogeneous forms of human chorionic gonadotropin and its free alpha subunit in the human choriocarcinoma cell line, JEG-3. Endocrinology 106: 849-858, 1980.

5. Kourides, I. A., Landon, M. B., Hoffman, B. J., and Weintraub, B. D.: Excess free alpha relative to beta subunits of the glycoprotein hormones in normal and abnormal human pituitary glands. Clin. Endocrinol. 12: 407-416, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45014-09 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Adrenal Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Wolff	Medical Director	CEB NIAMDD
	G. H. Cook	Chemist	CEB NIAMDD
	C. Londos	Senior Staff Fellow	LNE NIAMDD
	D. M. F. Cooper	Visiting Fellow	LNE NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.7	.2	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cell surface adenosine receptors mediate either stimulating or inhibition of adenylate cyclase activity, and the receptors that mediate these different responses can be discriminated with selected adenosine analogs. 5'-N-ethyl-carboxamide-adenosine is a more potent agonist at stimulatory receptors (R_a) than is N^6-phenylisopropyl-adenosine, whereas the reverse potency order is seen with inhibitory receptors (R_i). The potency of adenosine is intermediate between these two analogs. The relative potencies of adenosine receptor agonists is maintained in physiological responses in intact cells, such as steroidogenesis and inhibition of lipolysis. As with adrenergic receptors, subclasses of adenosine receptors differ functionally and pharmacologically.

Project No. Z01 AM 45014-09

Project Description:

The interaction of adenosine with adrenal cortical membranes in producing steroid secretion as well as interaction with other membranes has been further detailed by pharmacological investigation of stimulatory and inhibitory receptors associated with adenylate cyclases using dATP as substrate in the absence of adenosine deaminase. From a screening of numerous adenosine analogs, we have selected two which demonstrate the existence of subclasses of adenosine receptors: N^6-phenylisopropyladenosine (PIA) and 5'-N-ethylcarboxamide-adenosine (NECA). The relative potencies of the analogs in the adenylate cyclase studies are maintained in physiological studies on intact cells.

We conclude that distinct subclasses of adenosine receptors exist. Moreover, the receptor characteristics identified in adenylate cyclase studies in isolated membranes apply also to physiological responses in several intact cells. These data underline the predictive value of adenylate cyclase studies in the development of pharmacologic agonists or antagonists of the adenosine receptor. Alternatively, the type of adenosine receptor mediating a given response may be identified with the use of selected analogs in tissues containing several cell types and in which gross cyclic AMP changes may be misleading.

Publications:

1. Londos, C., Cooper, M. D. F. and Wolff, J.: Subclasses of external adenosine receptors. Proc. Natl. Acad. Sci. U.S.A. 77: 2551-2554, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45015-10 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Thyroid Plasma Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Wolff	Medical Director	CEB NIAMDD
	A. R. Goldhammer	Staff Fellow	CEB NIAMDD
	G. H. Cook	Chemist	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	.7	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Preactivation of beef thyroid membrane adenylate cyclase by Gpp(NH)p or F^- yields molecular weights of subsequently solubilized enzyme activity of 160,000 and 120,000, respectively. Both species of enzyme are still responsive to ADP-ribosylation with NAD by cholera toxin.

Project No.Z01 AM 45015-10

Project Description:

The molecular weight of NaF-activated, Triton N-101-solubilized adenylate cyclase from bovine thyroid membranes has been measured by a combination of sucrose density centrifugation and gel exclusion chromatography. The physical parameters are: sedimentation coefficient, 6.6S; Stokes radius, 41Å ; partial specific volume, 0.75 ml/g; molecular weight 119,000. This is in contrast to the molecular weight (159,000) of the enzyme from the same source activated with guanosine 5'-(β,γ-imido)-triphosphate.

In order to identify the missing protein of Mr ~ 40,000, attempts were made to see if this might be the guanine nucleoside regulatory protein.

Both soluble adenylate cyclase enzymes are subject to cholera toxin-mediated ADP-ribosylation. This implies that the diminished molecular weight of the NaF-activated solubilized adenylate cyclase is a consequence of one of the following: loss of a GTP binding protein and labeling of some other protein in the cyclase complex; loss of another protein not subject to cholera toxin labeling; or that two GTP binding proteins normally reside within the catalytic complex and upon NaF activation one is lost.

Publications:

Goldhammer, A., Cook, G. H., and Wolff, J.: Preactivation as a determinant for the size of thyroid adenylate cyclase. J. Biol. Chem. 255: 6918-6922, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45016-10 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Thyroid Hormone Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Wolff	Medical Director	CEB NIAMDD
	S. Berkowitz	Staff Fellow	CEB NIAMDD
	J. Fradkin	Clinical Associate	CEB NIAMDD

COOPERATING UNITS (if any)

Faculte de Medecine Alexis-Carrel, Lyon, France

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.6	.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lactoperoxidase binds to microtubules, ring oligomers and tubulin dimers with stoichiometries of 0.25, 0.6 and ~ 2.0, respectively, and an affinity constant of $2 \cdot 10^6$ M^{-1} for tubulin. Binding leads to dissociation of the α and β subunits to form native α-tubulin lactoperoxidases and β-tubulin lactoperoxidase.

Project Description:

In order to study secretion, tubulin-membrane interactions seem to be important. Thus, we attempted to label tubulin with ^{125}I by lactoperoxidase. Tubulin is damaged by iodination but, instead, we found that self-iodinated lactoperoxidase co-polymerizes with brain microtubules to constant specific activity and a stoichiometry of 0.2 to 0.3 lacteoperoxidase molecule/tubulin heterodimer polymerized in the presence of 4 M glycerol. By contrast, iodinated tubulin loses its competence to polymerize. The lactoperoxidase-microtubule association is salt- and temperature-sensitive, shows considerable specificity, is saturable, and is reversible. Lactoperoxidase does not displace the microtubule-associated proteins from microtubules, does not promote polymerization, and binds to preformed microtubules. Self-iodinated lactoperoxidase also binds to tubulin oligomers at 0°C and in the presence of $CaCl_2$. The stoichiometry for this interaction is 0.6 to 0.8 molecules of enzyme/dimer.

Lactoperoxidase forms a complex with soluble brain tubulin prepared by two cycles of polymerization and depolymerization by phosphocellulose chromatography. The interaction was studied by sucrose gradient analysis, gel filtration, and spectral analysis based on the finding that tubulin binding to lactoperoxidase leads to a red shift in the Soret spectrum, yielding a difference spectrum with a minimum of 410 nm and maximum at 430 nm. This interaction involves one or more sulfhydryl groups of insulin.

Complex formation is relatively slow, is retarded by 0.6 M NaCl, and is accelerated by diiodotyrosoine. By all three methods of analysis, the stoichiometry approaches a value of 2 lactoperoxidase molecules/tubulin dimer. There is a single class of binding sites in pig, beef, or rat tubulin with an apparent overall affinity constant of $\sim 2.0 \times 10^6\ M^{-1}$. The molecular weight of the complex by sucrose gradient or gel filtration is ~ 140,000, i.e. half of the expected value for a 2:1 adduct. Since both α and β subunits are present in the complex, we propose that the complex consists of a mixture of equal parts of presumably native α-tubulin-lactoperoxidase and α-tubulin-lactoperoxidase.

We have also studied the relation of Ca^{++} and calmodulin in the control of tubulin polymerization and eventually hope to relate this to secretion. Despite the supposed role of calmodulin-Ca^{++} interactions with tubulin, tubulin itself has intrinsic Ca^{++} binding sites of high and low affinity that vary with the state of polymerization or the pathway of polymerization. This interaction is being worked out before adding calmodulin to the control system.

Publications:

1. Rousset, B. and Wolff, J.: Lactoperoxidase-tubulin interactions. J. Biol. Chem. 255: 2514-2623, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45017-06 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Mechanism of Action of Lithium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Wolff	Medical Director	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive

Project No. Z01 AM 45017-06

Publications:

1. Wolff, J.: Lithium Interactions with the Thyroid Gland. In Cooper, T. B., Gershon, S., Kline, N. S. and Schou, M. (Eds.): Lithium - Controversies and Unresolved Issues, International Congress Series 478. Amsterdam, Excerpta Medica, 1979, pp. 552-564.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45018-05 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Adenylate Cyclase and Other Extracellular Products of B. Pertussis: Effects on Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Wolff	Medical Director	CEB NIAMDD
	A. Goldhammer	Staff Fellow	CEB NIAMDD
	S. Berkowitz	Staff Fellow	CEB NIAMDD
	H. G. Cook	Chemist	CEB NIAMDD
OTHER:	C. R. Manclark	Res. Microbiologist	FDA

COOPERATING UNITS (if any)

Division of Bacterial Products, Bureau of Biologics, FDA

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.3	.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The adenylate cyclase of *Bordetella pertussis* organisms is stimulated 100-1000 fold in a dose-dependent manner by calf-brain calmodulin. The system has the following properties: 1) The activation is prevented by EGTA and restored by calcium ion. 2) Oxidation of the methionine residues of calmodulin abolishes the ability to activite the cyclase. 3) Trifluoperazine inhibits calmodulin-activated cyclase. 4) A troponin C preparation stimulates the B. pertussis cyclase with < 0.01 the potency of calmodulin. This is the first report of a calmodulin effect in a prokaryote.

Project No. Z01 AM 45018-05

Project Description:

Adenylate cyclase of Bordetella pertussis has been shown to require a protein activator for optimum activity. Its remarkable heat stability suggested a certain resemblance to an activator of mammalian brain adenylate cyclases, namely, calmodulin (or calmodulin-dependent regulator). This small protein of 16,700 daltons interacts with a large number of enzymes and proteins in a Ca^{++}-dependent manner by a two-step mechanism that may be depicted as follows:

$$\text{Calmodulin} + Ca^{2+} \rightleftarrows \text{Calmodulin}\cdot Ca^{2+}$$

$$\text{Enzyme}_{\text{(inactive)}} + \text{Calmodulin}\cdot Ca^{2+} \rightleftarrows \text{Enzyme}\cdot\text{Calmodulin}\cdot Ca^{2+}_{\text{(active)}}$$

We find that calf brain camodulin stimulates adenylate cyclase activity of intact organisms 50-1000 fold, this is inhibited by EGTA and restored by excess Ca^{2+} and occurs at concentations of $\sim$ 10 nM camodulin for half maximal activation. Tropinin C is < 0.01 as active as is calmodulin whose methionine residues have been oxidized. Trifluoperazine inhibits the activation of the cyclase as it does other calmodulin-regualted enzymes. These data all suggest that calmodulin is an activator of cyclase in B. pertussis.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45020-05 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Synthesis of Thyroxine Transport Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
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	M. Berman	Senior Scientist	LTB NCI

COOPERATING UNITS (if any)

Veterans Administration Hsopital, Washington, D. C.; National Cancer Institute

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45021-04 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Pituitary Gonadotropic Pathophysiology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Rosen	Medical Director	CEB NIAMDD
OTHER:	J. M. Lieblich	Director, Med. Educ.	Highland Park Hosp., IL
	A. S. Rogol	Assoc. Prof. Ped.	Univ. Va. Sch. of Med., VA
	B. White	Sr. Investigator	LEP NIAMDD
	K. Mittal	Research Microbiologist	BB DBBP
	J. Morrow	Clinical Associate	CEB NIAMDD

COOPERATING UNITS (if any)
Highland Park Hospital, Department of Medicine, IL; University of Virginia School of Medicine, VA; Diabetes Unit, Beth Israel Hospital, Boston, MA

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.4	.4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies continue on syndromes of hypogonadotropic hypogonadism with anosmia (the Kallmann syndrome) and without anosmia, as well as on patients with anosmia who are clinically eugonadal. Analysis of eight families suggests genetic heterogeneity of the Kallmann syndrome.

PHS-6040

A. The Syndrome of Hypogonadism and Anosmia (the Kallmann Syndrome)

We have previously documented phenotypic (classic eunuch/fertile eunuch) and physiologic (normal/decreased response of circulating FSH and LH to GnRH) heterogeneity in patients with the Kallmann syndrome. Analysis of eight families suggests that the syndrome exhibits genetic heterogeneity as well.

Eight of 18 patients (44%) with the Kallmann syndrome (KS) had one or more relatives with either anosmia and hypogonadotropic hypogandism, anosmia alone, or cryptorchidism plus cleft lip and/or palate. Diabetes mellitus and obesity were frequent in probands, and 44% of patients had diabetic relatives. None of the patients had chromosomal abnormalities. There were no dermatoglyphic findings unique to KS, but the frequency of certain traits differed from mean values of the general population. In one kindred with six cases of KS, diabetes and cleft lip and palate occurred in both affected and unaffected persons; transmission of the syndrome was compatible with autosomal recessive inheritance but multifactorial inheritance could not be excluded. In several other families in which only a few males demonstrated full or incomplete expression of the syndrome, transmission could have been either X-linked or autosomal. Still other families had positive findings in both sexes, suggesting autosomal rather than sex-linked transmission. Our observations and those of some earlier investigators indicate that inheritance in some families is autosomal. These, together with previous reports of apparent X-linked transmission in other families suggest genetic heterogeneity of the Kallmann syndrome.

B. Familial Isolated Hypogonadotropic Hypogonadism

Detailed investigation of the hypothalamic-pituitary axis in three affected siblings revealed a deficiency of basal and GnRH-stimulated serum gonadotropins. A clinically eugonadal brother had normal serum testosterone, LH, and FSH, and a normal response to GnRH. HLA phenotyping revealed antigens common to the three patients (A3, BW 35, DRW1) but absent in their normal brother. These antigens were also different from that (A29) shared by each of two unrelated "fertile eunuchs" with the Kallmann syndrome.

C. Eugonadal Congenital Anosmia

Detailed studies of hypothalamic-pituitary function have been performed on a 24-year-old phenotypically eugonadal man with congenital anosmia. No deficits were revealed other than his known inability to smell. We are trying to recruit other patients for this study.

Publications:

1. Rogol, A. D., and Rosen, S. W.: Heterogeneity of response of serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to gonadotropin-releasing hormone (GnRH) in patients with the syndrome of congenital anosmia and hypogonadotropic hypogonadism. In Beling, C. and Wentz, A. (Eds.): The Luteinizing Hormone-Releasing Hormone. New York, Masson, 1980, pp. 237-243.

2. Rogol, A. D., Mittal, K. K., White, B. J., McGinniss, M. H., Lieblich, J. M., and Rosen, S. W.: HLA-compatible paternity in two "fertile eunuchs" with congenital hypogonadotropic hypogonadism and anosmia (the Kallman syndrome). J. Clin. Endocrinol. Metab., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45023-04 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

TSH Binding Sites: Relationship to Anti-thyroid Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. D. Weintraub	Medical Director	CEB NIAMDD
OTHER:	F. Pekonen	Visiting Associate	CEB NIAMDD
	P. Carayon	Guest Worker	ERRB NICHD

COOPERATING UNITS (if any)

Endocrinology and Reproduction Research Branch, NICHD

LAB/BRANCH
Clinical Endocrinoloy Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	.8	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Preincubation of human and porcine thyroid membranes in high concentrations of ammonium sulfate resulted in an increase in high affinity TSH receptors without change in low affinity receptors. Various forms of intracellular and extracellular TSH differing in carbohydate content displayed different receptor-binding and adenylate cyclase activities. Lymphapheresis caused a major decrease in serum thyroid-binding immunoglobulins in 2 patients with Graves' Disease.

PHS-6040

Project No. Z01 AM 45023-04

Project Description:

I. Salt-Induced Exposure of High Affinity Thyrotropin Receptors

We have previously demonstrated that the predominant TSH binding site in thyroid plasma membranes has relatively low affinity and specificity. Another binding site of high affinity and specificity for TSH is present in smaller amounts, and near physiologic incubation conditions are required for its detection. The relatively low amounts of the high affinity receptor has impeded studies of its regulation and efforts to purify this site.

We thought it possible that the high affinity receptor was more exposed in the intact thyroid gland but became disrupted or "masked" during homogenization and membrane purification. Therefore, we examined several new methods of membrane isolation including the use of protease inhibitors aprotinin and phenyl methyl sulfonyl fluoride (PMSF). In addition, we found that preincubation of human and porcine thyroid membranes in high salt concentration before binding studies resulted in a 4 to 8 fold increase in high affinity TSH receptors (affinity constant = 10^{10}) without any change in low affinity receptors ($K = 10^{7}$). Salt-induced exposure of high affinity receptors was pH and temperature dependent, and was maximal at pH 5.0, 37°C in the presence of 1 M $(NH_4)_2SO_4$. The exposure of these sites could be blocked by prior trypsin treatment suggesting an alteration in exposed proteins.

The new receptors exposed by salt were indistinguishable in affinity or specificity from those present on membranes before treatment. They showed no interaction with > 1000-fold excess of gamma globulin, thyroglobulin, ACTH, cholera toxin and gangliosides. In contrast, the low affinity receptors, which were unchanged by salt, continued to show interaction with these non-thyrotropic substances.

These studies provide further support for the existence of two separate types of TSH binding sites. The methods developed will be applied toward a further characterization of the high affinity site, which we believe is the receptor mediating the biologic actions of TSH.

II. Effect of Carbohydate on TSH-Receptor Interactions and Biologic Action

We have examined the role of carbohydrate in TSH-receptor interaction and biologic action. Previous studies from our laboratory have indicated that deglycosylation of thyroid membranes had little effect on high-affinity TSH binding. Subsequently, we were able to deglycosylate TSH only partially by exoglycosidases. However, by culturing mouse thyrotropic tumor cells in the presence of tunicamycin, we produced carbohydrate-free α and β subunits that did not

combine to form TSH. These experiments suggested that carbohydrates were necessary for subunit interaction and ultimate biologic action (see also Z01 AM 45013-08 CEB for effects of tunicamycin on labeled subunits).

We also examined several forms of intracellular and extracellular TSH produced by thyrotropic tumor cells that appear to differ in carbohydrate content. After separation on long (160 cm) columns of Sephadex G-100, we were able to test the immunoactivity, receptor-binding properties, and adenylate cyclase activity of each form of TSH. We found three distinct molecular weight forms of TSH with widely different ratios of receptor activity/immunoreactivity as well as biologic activity/immuoreactivity. We are attempting to characterize the carbohydrate differences among these forms and to selectively remove sugars from each form and retest their properties.

III. Effect of Cytopheresis on Thyroid-Binding Immunoglobulins

We have initiated a randomized study for the treatment of Graves' ophthalmopathy with either lymphapheresis or plasmapheresis. To date we have treated 4 such patients with 6 week courses (2 procedures/week) of either procedure. Patients have demonstrated some subjective improvement (possibly attributable to a placebo effect) but only equivocal objective changes. The lack of objective improvement may be related to the long duration of disease in each patient, associated with irrevocable fibrosis.

Thyroid-binding immunoglobulins (TBI) have been evaluated in 3 patients who received lymphapheresis. One patient had no detectable TBI at any time. However, in the other 2, high levels of TBI were reduced to normal during the procedure and remained normal for at least 2 months after the last procedure. These studies suggest that, in properly selected patients (i.e., recent onset), the procedure could affect the course of the disease. Even if ophthalmopathy is not affected, it is possible that lymphapheresis could produce a remission in Graves' hyperthyroidism. Perturbations of TBI in these patients will allow a crucial test of the hypothesis that such humoral antibodies are the cause of the hyperthyroidism.

Publications:

1. Pekonen, F. and Weintraub, B. D.: TSH receptors on bovine thyroid membranes: Two types with different affinity and specificity. Endocrinology 105: 352359, 1979.

2. Pekonen, F. and Weintraub, B. D.: Interactions of crude and pure chorionic gonadotropin with the thyrotropin receptor. J. Clin. Endocrinol. Metab. 50: 280-285, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45024-04 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Metabolism of Thyroid Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Sato	Visiting Associate	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
	K. Sorimachi	Visiting Associate	CEB NIAMDD
OTHER:	R. D. Sekura	Staff Associate	LBM NIAMDD
	W. B. Jakoby	Chief, Enzymes and Cell. Biochem. Sect.	LMB NIAMDD

COOPERATING UNITS (if any)
Endocrine-Metabolic Service, Walter Reed Army Medical Center, Washington, D.C.

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.2	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The relation of glutathione to thyroid hormone metabolism was studied in cultured monkey hepatocarcinoma cells and normal rat hepatocytes. In carcinoma cells, the major deiodination of thyroxine affects the non-phenolic ring yielding reverse triiodothyronine whereas, in normal hepatocytes, it is phenolic ring deiodination yielding triiodothyronine. By culturing cells in cystine- and/or methionine-deficient medium glutathione was reduced below 5% of control, but no decrease in deiodination was observed; therefore, the lesser variations observed in previous studies probably do not regulate deiodination. Transfer of sulfate to thyroid hormones and analogs was studied with monkey hepatocarcinoma cell extract and 2 homogeneous aryl sulfotransferases from rat liver. The differences in sulfation explain the relative sulfation of these compounds in vivo and in living cells. Metabolism of [3',5'-^{125}I] and [3,5-^{125}I]diiodothyronine and thyroxine in monkey hepatocarcinoma cells was studied. Various monodeiodination reactions were detected, but no diphenyl ether splitting was observed.

Project Description:

1. It is generally believed that non-protein sulfhydryl groups mainly glutathione (GSH), are essential for monodeiodination of thyroid hormones and that the observed decrease in non-protein sulfhydryl groups (30-50% of control) accounts at least in part for the decreased conversion of T_4 to T_3 in starved or fetal rat liver. We investigated this question by inducing marked glutathione deficiency in hepatocyte monolayers using continuously cultured monkey hepatocarcinoma cells and primary cultures of normal adult rat hepatocytes. To decrease intracellular glutathione, cells were grown for 20 to 40 h in medium deficient in cystine and/or methionine. In hepatoma cells control glutathione, 29.6 $\pm$ 5.3 µg per mg of protein, fell to 1.7 $\pm$ 0.2 in Cys(-) medium and 1.3 $\pm$ 0.5 in Cys(-)Met(-) medium. In normal hepatocytes, control glutathione 15.4 $\pm$ 3.8, was unchanged in Cys(-) medium but fell to 1.1 $\pm$ 0.1 in Cys(-)Met(-) medium. Metabolism of ^{125}I-labeled thyroxine, 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine (reverse T_3) was studied by thin-layed chromatography. As reported previously, hepatoma cells were highly active in 5-deiodination whereas the rat hepatocytes were highly active in 5'-deiodination. No decrease in deiodination was observed in glutathione deficient cells of either type. Further decrease of sulfhydryl groups by diamide indicated that they are required for deiodinase activity. These data, however, indicate that marked variations in glutathione concentration do not regulate the deiodination pathways.

2. Phenolic conjugation of the thyroid hormones with sulfate is a detoxification reaction which occurs primarily in the liver. The transfer of sulfate to thyroid hormones and their analogs was studied in monkey hepatocarcinoma cell extract and was compared to their reaction with two homogeneous aryl sulfotransferases from rat liver. While the thyroid hormones, thyroxine and triiodothyronine, and their analogs varied widely in their capacity as sulfate acceptors, the catalytic ability of these 3 enzyme preparations generally reflected a broad pattern of overlapping specificity. It was found that thyroxine is a very poor substrate, that 3'-iodothyronine and 3,3'-diiodothyronine are very good substrates, and that 3,5,3'-triiodothyronine is intermediate in its acceptor activity. This probably explains the relative sulfation of these compounds *in vivo* and by living hepatocytes.

3. Cultured monkey hepatocarcinoma cells were incubated with [3',5'-^{125}I]-diiodo-L-thyronine ([3',5'-^{125}I]T_2) and with [3,5-^{125}I]diiodothyronine ([3,5-^{125}I]T_2). Monodeiodination as well as sulfoconjugation took place in both cases. [3'-^{125}I]iodothyronine ([3'-^{125}I]T_1) and [3',5'-^{125}I]T_2 were identified as metabolites of outer ring-labeled T_4 in the cells, but neither [3-^{125}I]iodothyronine ([3-^{125}I]T_1) nor [3,5-^{125}I]diiodothyronine ([3,5-^{125}I]T_2) was detected after incubation of the cells with inner ring-labeled T_4.

No products of diphenyl ether splitting were observed after incubation of the cells with ether [3,5-^{125}I]T_2 or [3,5-^{125}I]T_4.

4. A radioimmunoassay for 3,5-diiodo-L-thyronine (3,5-T_2) has been devised. It has a lower limit of detectability of 0.5 ng/dl. Using this RIA, it was found that 3,5-T_2 circulates in the serum of normal subjects, is increased in hyperthyroidism and decreased in hypothyroidism. The serum 3,5-T_2 level depends on and is probably derived from circulating T_3. Fasting did not seem to inhibit deiodination of T_3 to 3,5-T_2 as it does that of T_4 to T_3.

Publications:

1. Sorimachi, K., and Robbins, J.: Phenolic and nonphenolic ring deiodinations of iodothyronines in cultured hepatocarcinoma cell hemogenate from monkey. Biochem. Biophys. Acta 583: 443-453, 1979.

2. Sorimachi, K., and Cahnmann H. J.: Formation and metabolism of 3',5'-diiodothyronine and 3,5-diiodothyronine by cultured monkey hepatocarcinoma cells. Horm. Metab. Res. 11: 233-237, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45025-02 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Effects of Triiodothyronine on Liver Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. E. Rall	Director, Intramural Research Programs	ODIRP NIAMDD
	V. Nikodem	Staff Fellow	CEB NIAMDD
	S.-Y. Cheng	Research Chemist	CEB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Protein Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The thyroid hormone receptor from rat liver nuclei was covalently labeled with the N-bromoacetyl derivatives of L-thyroxine (T_4) and 3,3',5-triiodo-L-thyronine (T_3). SDS gel electrophoresis of the labeled nuclear receptor showed one major radioactive component with a molecular weight of 56,000. We have been studying qualitative and quantitative changes of rat liver nuclear proteins in control rats, thyroidectomized and thyroidetomized after triiodothyronine (T_3) injection. The most profound change was found in a protein of molecular weight 32,000 D. This protein has the highest relative intensity in control, lowest in hypothyroid rats and its intensity gradually increases after 6, 12, 18 and 24 hours after T_3 injection, when it reaches 50% of the control level. Because of our interest in an effect of thyroid hormones on chemical changes of nuclear proteins, we studied the acetylation of the crude preparation of HMG proteins in control and hypothyroid rats. Three major peaks in a range of molecular weight 18-12,000 D were highly acetylated. At the present time, they have not been conclusively identified, but they co-migrate with the mixture of calf thymus histones.

PHS-6040

Project Description:

1. The thyroid hormone receptor from rat liver nuclei has been covalently labeled with the N-bromoacetyl derivatives of L-thyroxine (T_4) and 3,3',5-triiodo-L-thyronine (T_3). Displacement binding studies showed that in the presence of 100-1000 molar excess of unlabeled N-bromoacetyl T_3 or T_4, binding of [^{125}I]T_3 or [^{125}I]T_4 was inhibited, respectively. This indicated that T_3 or T_4 and its bromoacetyl derivatives compete for the same binding site. The nuclear receptor showed identical behavior in high pressure liquid chromotography (HPLC) whether bound with T_3, T_4 or covalently labeled with their bromoacetyl derivatives. HPLC provided a single step 100 fold purification of the nuclear receptor. SDS gel electrophoresis of the nuclear receptor labeled with N-bromoacetyl derivatives of [^{125}I]T_3 or T_4 showed one major radioactive component with a molecular weight of 56,000. Furthermore, in the absence of denaturant, the nuclear receptor either bound with [^{125}I]T_3 or covalently labeled with N-bromoacetyl [^{125}I]T_3 showed identical mobility. These results suggested that the nuclear receptor is a single polypetide chain and binds either T_3 or T_4. Nuclear receptors covalently linked with N-bromoacetyl derivatives of [^{125}I]T_3 or T_4 may be useful as marker for the preparative purification of receptor.

2. It has been proposed that chromatin-associated proteins play a role in regulating the pattern of gene expression in higher organisms. In some cases, changes in chemical structure of nuclear proteins appear to be under hormonal control.

   We have been studying qualitative and quantitative changes of rat liver nuclear proteins in control rats, thyroidectomized and thyroidetomized after triiodothyronine (T_3) injection. Hypothyroid rats were intravenously injected with T_3 (1 mg/100g body weight) and liver nuclear proteins were analyzed after 6, 12, 18, 24 hours and 3 days after the injection. To achieve a good reproducibility, it was necessary to extract nuclear proteins in reproducible manner. The combination of 8M urea, NP-40 and 2% SDS gave us in most experiments satisfactory results. The techniques of isoelectrical focusing (IEF) and SDS electrophoresis were used two-dimensionally and proteins were stained with Coomassie brilliant blue R250. To avoid smearing in the second dimension, the ratio of NP-40/SDS had to be kept 1:8. When ampholine of pH 3.5-10 was used and IEF was performed for 6800 V h, pH gradient 4-7.5 was formed and acidic proteins were well resolved. We observed differences in protein pattern in the basic end of the pH gradient together with large amount of proteins which did not enter the gel. Therefore, nonequilibrium pH gradient electrophoresis towards cathode for 1500 V h was employed.

   The gels are analyzed by computer in collaboration with Dr. Benes Trus (NIDR). First, the gel is photographed on 4x5 linear film, which is scanned on the Perkin-Elmer microdensitometer. The computer program

locates the peak and finally integrates each peak. With this technique, over 500 spots were resolved. The next step is to compare two 2-D gels with each other and to determine where peaks differ. This is done by program overlap. So, if the gel from control experiment is used to overlap with gels of hypothyroid or hypothyroid rats after T_3 injection qualitative and quantitative changes are obvious. At the present time, these changes have been analyzed.

The most profound change was found in a protein of molecular weight 32,000 D. This protein has the highest relative intensity in control, lowest in hypothyroid rats and its intensity gradually increases after 6, 12, 18 and 24 hours after T_3 injection, when it reaches 50% of the control level (4.15; 0.057; 1.66; 1.71; 1.86; 2.11). This change was detected after 6800 V h of IEF and also after 1500 V h. However, after three days the relative intensity of this protein reached the level of one found in hypothyroid rats.

3. A group of nonhistones chromosomal proteins called the high mobility group proteins (HMG) became the object of extensive studies in recent years in the search for nuclear proteins which might serve more specific functions. They can be extracted from whole tissue by 0.2 M H_2SO_4 together with some histones. They are soluble in 2% TCA and insoluble in 10-20% TCA. Recent data have indicated that actively transcribing chromatin fractions are enriched in HMG proteins relative to inactive chromatin. These proteins can be also easily acetylated.

Because of our interest in an effect of thyroid hormones on chemical changes of nuclear proteins, we studied the acetylation of the crude preparation of HMG proteins in control and hypothyroid rats. Liver homogenates were incubated in the presence of ^3H sodium acetate, HMG protein were prepared as above and this crude preparation, which also contains histones, was run on 10-20% SDS gel gradient. The gels were cut and incorporation of acetate was measured at Beckman scintillation counter after gel pieces were dissolved in 30% H_2O_2 and 10 ml of ultrafluor was added. Very low acetylation was observed for H1 and HMG 1 and 2. However, 3 major peaks in a range of molecular weight 18-12,000 D were highly acetylated. At the present time, they have not been conclusively identified, but they co-migrate with the mixture of calf thymus histones.

The preparation from hypothyroid rats incorporated 179% more acetate than preparations from control rats. When short IEF electrophoresis was performerd, one major radioactive peark with two shoulders was observed at pH 8 for acetylated proteins. If the degree of acetylation was less, the radioactive peak after IEF shifted 0.2 pH units to pH 8.2.

Butyrate did not enhance acetylation (as had been expected) probably because the incubation times were short compared to the rate of deacetylation.

To verify that acetylated proteins are nuclear proteins, nuclei were prepared after the whole liver homogenate was incubated with ^{3}H sodium acetate. Proteins were then extracted with 8 M urea and 2% SDS and run on 10-20% SDS gel gradient. The positions of radioactive peaks correspond to those obtained from whole homogenate using 0.2 M H_2SO_4 and 20% TCA precipitation.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45026-02 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Model Reactions for the Synthesis of Thyroxine Residues in Thyroglobulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
	J.-M. Gavaret	Visiting Associate	CEB NIAMDD

COOPERATING UNITS (if any)
Unitè de Recherche sur la Glande Thyroide et la Règulation Hormonale, INSERM, Bicetre, France

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45027-02 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Synthesis of Thyroid Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
	K. Sato	Visiting Associate	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.4	.3	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A thin-layer chromatographic method of analyzing triiodo-L-thyronine (T_3) has been devised. A slight modification of this method permits its use for preparative purposes.

Project No. Z01 AM 45027-02

Project Description:

The synthesis of triiodo-L-thyronine (T_3) of high specific activity, labeled exclusively in the nonphenolic ring reported last year has been perfected through the use of an acidic solvent system that permits far better separation of T_3 from contaminating diiodo-L-thyronine and thyroxine by thin-layer chromatography than the solvents customarily used for this purpose. A slight modification of this method permits its use for preparative purposes.

Publications:

1. Sato, K. and Cahnmann, H. J.: Synthesis of [3,5-^{125}I]triiodo-L-thyronine of high specific activity. Anal. Biochem. 102: 237-242, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45028-02 CEB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Thyroid Hormones-Cell Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.-Y. Cheng	Research Chemist	CEB NIAMDD
	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
OTHER:	I. Pastan	Chief, LMB	LMB, NCI
	M. Willingham	Senior Surgeon	LMB, NCI
	J. Maxfield	Visiting Fellow	LMB, NCI
	R. Johnson	Research Chemist	CEB, NIAMDD

COOPERATING UNITS (if any)

National Cancer Institute

LAB/BRANCH
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Endocrine Biochemistry

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using video intensification fluorescence microscopy and rhodamine-labeled triiodothyronine, the uptake of T_3 by cultured mouse fibroblasts was investigated. A saturable sequestering of labeled-hormone on the cell surface was observed leading to the formation of endocytic vesicles. By comparison with the entry of α^2-macroglobulin into the same cells it was concluded that thyroid hormone was accumulated and endocytosed through coated pits.

Project No. Z01 AM 45028-02

Project Description:

It is uncertain whether thyroid hormones enter cells by simple diffuion or by some other mechanism. Using video intensification fluorescence microscopy and rhodamine-labeled 3,5,3'-triiodo-L-thyronine (Rho-T_3), uptake of T_3 was studied in cultured mouse fibroblasts. After incubation of cells with Rho-T_3 for 30 min. at 37°C, the fluorescent hormone was concentrated in many small bright accumulations. This phenomenon was prevented prevented by excess T_3 and was not seen with rhodamine itself or rhodamine-labeled thyronine. It also did not occur at low temperature. Following this receptor-mediated adsorbtion to the cell surface, Rho-T_3 was seen in endocytic vesicles. It was shown that this entry process was identical with that traversed by α2-macroglobulin and therefore is related to the coated pits on the cells surface. The clustering and internalization phenomenon, but not the binding, were inhibited by methylamine, dansylcadaverine or bacitracin. This is the first demonstration of a low molecular weight hormone entering cells by the coated pit pathway. The endocytic vesicles do not appear to combine with lysosymes, and this mode of entry may be at least one mode of passage of thyroid hormones to their intracellular receptor sites.

In related studies using cultured monkey hepatoma cells, it was shown that Rho-T_3 incubated with cell monolayers is accumulated by the cell nucleus in a specific and saturable manner. The mode of entry of the hormone into the hepatocyte will be examined in future studies.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45029-01 CEB

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Hyperpigmentation-Gammopathy Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Rosen	Medical Director	CEB NIAMDD
OTHER:	J. Morrow	Clinical Associate	CEB NIAMDD
	D. Huston	Clinical Associate	ARB NIAMDD
	E. Schaefer	Sr. Investigator	MDB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Physiology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are investigating the mechanism of hyperpigmentation and hyperprolactinemia in a woman with benign monoclonal gammopathy.

PHS-6040

Project No. Z01 AM 45029-01

Project Description:

We are studying a 46-year-old white woman of Italian ancestry. She has had hyperpigmentation and (benign) monoclonal, kappa light chain gammopathy for more than a decade. Four years ago, galactorrhea appeared and hyperprolactinemia was discovered; trans-sphenoidal surgery revealed no evidence of pituitary tumor and the hyperprolactinemia has persisted. A handful of similar cases has been published, notably by Imawari et al. (Ann. Int. Med. 81: 490, 1974). These patients differed from ours in the presence of polyneuropathy and hepatomegaly; their prolactin status was not reported, although other endocrinopathies (gynecomastia, glucose intolerance, anrenorrhea, hirsuitism) were noted in some cases.

We are looking for the answers to three questions in our patient: 1) Will plasmapheresis decrease the serum level of her monoclonal IgG and will this be accompanied by a decrease in pigmentation. 2) Does the monoclonal IgG stimulate melanin production. 3) What is the etiology of her hyperprolactinemia.

Thrice weekly plasmaphereses were carried out for a month. The patient's total gamma globulin fell from 2.22 to 0.46 g/dl; quantitative determinations of IgG are pending. She is now off plasmapheresis and frequent serum samples are being obtained to follow the increasing levels of her monoclonal IgG.

Melanin production by melanoma cells in vitro is being measured by Dr. Abla Creasey. These determinations are being done basally as well as with the patient's and control IgG's added to the culture medium. Dr. Y. P. Loh is looking at the effects of the patient's and control IgG's on stimulation of melanocytes in chameleon (Anolis carolinensis) skin.

The patient's IgG did not stimulate prolactin production by Dr. Armen Tashjian's line of TRH-responsive rat pituitary tumor cells (GH_3). Gel chromatography of the patient's serum revealed that her circulating prolactin is heterogeneous, with the dominant fraction having an apparent mol wt of 210,000; since co-chromatographed ^{125}I-prolactin migrated as a monomer (app. mol wt 17,000), it is unlikely that the patient's monoclonal IgG was binding her endogenous prolactin and raising the serum concentration by decreasing its clearance. We and others have observed large forms of circulating prolactin in other patients; the nature of the macromolecular prolactin in this patient is being investigated.

Publications:

None

ANNUAL REPORT 1979 - 1980
Diabetes Branch, NIAMDD

INSULIN AND INSULIN RECEPTORS

Work in this area has been recognized by several awards (a) The First Lita Annenberg Hazen Award For Excellence in Clinical Research ($100,000); (b) Public Health Service Distinguished Service Medal; (c) Honorary Professorship at the University of Uppsala, Uppsala, Sweden; (d) Solomon A. Berson Award from the American Diabetes Association, Inc.; and (e) grant awards from the Kroc Foundation, American Diabetes Association, Inc., Washington, D. C. Affiliate and the Juvenile Diabetes Foundation.

Insulin in Brain and Other Organs: We have previously shown that the brain and most other extrapancreatic tissues as well as cells grown in tissue culture contain material that is indistinguishable from insulin at concentrations equal to or many fold higher that that found in plasma or other fluids in which the cells are maintained. Extreme changes in the concentration of insulin in the medium or in the blood had little or no effect on the concentration of insulin extracted from the cells. We have, therefore, raised the possiblility that most or all cells produce small amounts of insulin.

To show more clearly that the β-cells of the pancreas do not have a monopoly on insulin production, we studied flies and worms which are known not to have a pancreas. Acid ethanol extracts of both the flies and worms contained insulin at several ng/gm which was indistinguishable by multiple criteria from typical mammalian insulins. In addition, we have studied chick embryos. In the developing chick embryo the pancreas does not develop islet cells until days 5-7. Our findings of insulin in chick embryos at earlier times further supports the idea that insulin production is not a property unique to β-cells of vertebrate pancreases. (Le Roith, Lesniak, Pruss, de Pablo)

Studies in the guinea pig have permitted us to distinguish clearly between pancreatic and cellular insulins. Guinea pigs are known to have in their pancreas an insulin which is quite unusual in several respects. About 1/3 of its amino acids are different from those of most mammalian insulins, its biological activity is reduced to about 5% of normal, even in the guinea pig, and it reacts hardly at all with antibodies directed against mammalian insulins. In the present study, using specific assays for mammalian insulin and guinea pig insulin, we have shown that brain and extrapancreatic tissues of the guinea pig contain a typical mammalian insulin at concentrations similar to those found in the tissues of rodents and humans. The insulin in the brain and other extrapancreatic tissues is indistinguishable from other mammalian insulins. This is in contrast to the insulin found in the pancreas and plasma of these animals which is the usual guinea pig insulin. These data provide the strongest evidence that cellular insulin is not derived from the pancreas via the plasma but rather is of local extrapancreatic origin. In addition it suggests that the evolution of insulin, at least in the guinea pig, is non-allelic i.e. we propose that the gene for guinea pig insulin did not mutate recently but rather there was a

duplication of the primordial insulin gene millions of years before the origin of mammals and that the late mutational event was that the pancreas over-produces the product of this mutant gene while the brain and other extra-pancreatic tissues continue to express the typical mammalian insulin gene. (Rosenzweig, Samuels)

Insulin Receptors on Circulating Cells and Cultured Cells: We and others have shown that insulin receptors are frequently involved in disorders of glucose metabolism and insulin sensitivity. To study insulin receptors in humans, fresh from the in vivo, we have used circulating monocytes and erythrocytes; to study the intrinsic properties of insulin receptors in individual patients outside of the in vivo environment, we have used cultured fibroblasts and most recently transformed B-lymphoctyes. B-lymphocytes from normal subjects as well as from a range of patients with disorders of glucose metabolism were exposed to Epstein-Bar virus which allows the establishment of permanent B-cell lines. B-cell lines have insulin receptors and we have shown that two patients with extreme insulin resistance and severe defects of insulin binding to its receptors in vivo express severe deficiencies of insulin receptors on their cells grown in culture. In both patients, the number of insulin receptors was markedly reduced but the residual receptors where normal by multiple criteria. (Taylor)

Fibroblast have been cultured and their insulin receptors have been studied in vitro in several disorders. In addition, the activation of glycogen synthase, an important biological action of insulin, has been studied. Other projects using fibroblasts have been introduced not only to study the metabolic effect of insulin such as enzyme activation and induction and transport of small molecules but also to study the growth promoting effect of insulin. (Podskalny)

Techniques have been recently worked out to study insulin receptors on erythrocytes. These cells are much more convenient to obtain from young patients and much less blood is required, thus, providing a cell in which to study receptors in young patients. Young erythrocyes have more insulin receptors than older erythrocytes. Studies have been completed which allow an assessment of cell age (by measurements of enzyme levels in the cells) and thereby to correct measures of insulin binding to factor out the role of cell age independent of other factors such as the metabolic state for disease conditions of the patient at the time when the studies are done. (Dons)

Patients with insulin dependent diabetes as well as patients with glucocorticoid excess have had insulin binding to their receptors on red cells and monocytes evaluated as a function of the progress of their primary illness and their metabolic or glycemic state. (Fantus, Ryan, Saviolakis)

3T3-L1 fibroblasts represent a cell line that converts from a fibroblast to a fatty cell which has many of the characteristics of normal adipose tissue. In previous studies, we had shown that chronic glucocorticoid excess in vivo produced characteristic changes in insulin binding to its receptors on liver. In the present study these changes were reproduced by adding glucocorticoids for several days to the 3T3-L1 cells in culture. That the

in vivo effect could be reproduced in simple in vitro systems should enable us to work out the cellular mechanisms in more detail. (Grunfeld, Baird)

Growth Promoting Effects of Insulin: Recent studies from this group have indicated that insulin and the insulin-like growth factors exert their metabolic properties through the insulin receptor, whereas both types of hormones exert their growth promoting effects through other receptors. Both sets of receptors only bind insulin and insulin-like growth factors but show a marked difference in their affinity and their relative preferences for insulin and insulin-like peptides. In the present work, more than 25 different insulins and chemically modified insulins were studied for their metabolic potencies and for their ability to stimulate cell division. Surprisingly, the growth promoting property of the insulin and insulin-like growth factors were all within a narrow range, whereas the metabolic potencies ranged over a thousand-fold. These data suggest that the growth promoting properties of insulins and insulin-like peptides are more highly conserved evolutionarily than is the metabolic affect. (King, Kahn)

Insulin and the insulin receptor not only interact with one another at the level of the receptor but show interactions at multiple other levels biologically. For example, insulin deficiency causes a marked reduction in circulating levels of the insulin-like growth factors. In a recent study, it was found that insulin binding to the insulin receptor increases the affinity of the growth factor receptor for its peptides by a mechanism that would fall under the heading of positive cooperativity. Thus, insulin not only acts permissively to permit the production of somatomedins but also enhances the effectiveness of any given circulating level of somatomedin by increasing the affinity of its specific receptors.

Previous studies from this laboratory had indicated that a substantial minority of patients with non-islet cell tumors associated with hypoglycemia were over-producing an insulin-like growth factor (probably IGF II). Another large series of these patients have been studied and have confirmed the early results in detail. In addition, work from another laboratory has provided confirmation of these studies. (Gorden, Hendricks)

Morphological Correlates of Insulin and Other Hormone Binding to Its Receptors: The reversible binding of insulin to its receptor on the cell surface is only the initial step in the hormone receptor interaction. Among subsequent events is the movement of hormone and receptor on the cell in multiple ways e.g. receptors can move from the villae to the smooth surface, receptors may be concentrated in coated pit regions of cells, the hormone-receptor complex may be internalized and transferred to Golgi type vesicules for the specialized regions near the cell surface, etc. In order to determine which of these processes are specific for the ligand and specific for the cell type, multiple ligands and multiple cell types have been studied.

In the present studies the interaction of ^{125}I-hGH has been studied in detail in vitro and has been correlated with morphological parameters such as quantitative EM autoradiography. In addition to insulin and growth

hormone, similar studies have been carried out with epidermal growth factor and with low density lipoproteins (LDL). The role of lysosomes in determining the fate of bound hormone and bound receptor is being examined in detail. (Lesniak, Hizuka, Van Obberghen, Gorden)

The interaction of ^{125}I-insulin with the 3T3-L1 fatty fibroblasts has been studied using EM autoradiography which showed that binding to the plasma membrane is followed rapidly by preferential association to the coated plasma membrane vesicules or coated pits. Further studies have been done to use other markers such as colloidal gold albumin, ferritin, and peroxidase. (Grunfeld, Van Obberghen)

Characterization of the Insulin Receptor: The insulin receptor is not available in large quantities in pure form. Therefore we have used the technique of radiation inactivation which permits the study of complex molecules in their natural environment in the impure state. Previous studies have indicated that high energy radiation produces an increase in the affinity in the insulin receptor presumbably by the destruction of a high molecular weight affinity regulator component. The regulator component appears not to have any insulin binding sites but rather acts to diminish the affinity of the binding sites for insulin. Recent studies have shown that the characteristic changes of the insulin receptor are unique to insulin and not found with receptors for epidermal growth factor or multiplication stimulating activity (MSA); the latter effect on receptors appears to be a simple inactivation i.e. loss of receptor sites with no change in affinity. The studies of the insulin receptor have been extended from plasma membranes of liver to those of IM-9 lymphocytes, rat adipocytes, and solublized receptors from rat liver. In addition, an extensive computer analysis of the data have yielded a working model for the molecular components of the insulin receptor complex. (Harmon)

Autoimmunity and Receptors: This laboratory originally described the existence of autoantibodies to the insulin receptor in patients with a syndrome of extreme insulin resistance and the skin disorder acanthosis nigricans. We have continued to study patients with this disorder and have developed a number of screening assays for the detection of receptor autoantibodies. Serum samples are sent to our laboratory for routine screening. These antibodies specifically inhibit insulin binding, immunoprecipitate solubilized insulin receptors, and mimic most of the acute and chronic metabolic effects of insulin. Recently we have demonstrated directly that the antibodies recognize the insulin binding subunit of the insulin receptor. The patients with insulin receptor antibodies appear to fit into the spectrum of autoimmune disease and have been treated with immunosuppressive agents and/or by plasma exchange. (Harrison)

Recently we discovered that the New Zealand obese (NZO) mouse, previously considered to be a model for obesity and insulin resistant diabetes, has autoimmunity with circulating IgM, antibodies to DNA and insulin receptors. These animals also develop neural lesions resembling those seen in both systemic lupus erythematosis and diabetes, and associated with the deposition of IgM on the glomenular basement membrane. Further studies will be performed in NZO mice and elucidate the role of animal factors in the pathogenesis of diabetic complications. (Harrison, Underhill)

In conjunction with Dr. C. J. Venter of Buffalo, N.Y., we have extended the concept of receptor autoimmunity to include autoantibodies to the beta-adrenergic receptor in some patients with asthma and allergic disorders. We are currently engaged in studies to determine the prevalence, pathogenetic significance, and mechanism of action of these antibodies. (Harrison)

Application of Insulin Receptor Autoantibodies: Autoantibodies have been used to purify and characterize insulin receptors. These antibodies bind to and immunoprecipitate the insulin binding sites within an oligomeric receptor. The receptor has a subunit structure consisting of at least two 126,000 Dalton recognition sites, and two associated 90,000 Dalton subunits whose function is unknown. These subunits have been identified in cultured lymphocytes by surface labeling with ^{125}I or biosynthetic labeling with ^{35}S-methionine, followed by immunoprecipitation with receptor antibodies. They have also been quantitatively purified by sequential chromatography on wheat germ lectin and receptor antibody columns. (Hedo, Kasuga, Van Obberghen)

The receptor autoantibodies have been used to measure the receptor molecule by radioimmunoassay, to show that "down-regulation" of receptor function involves loss of receptors from the cell surface, and to show that a fraction of insulin bound to cells is internalized as a complex with its receptor. (Kasuga)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 47,000 - 06 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cooperative Mechanisms Regulating Receptor's Affinity for Insulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. Van Obberghen	Visiting Associate	DB, NIAMDD
	P. De Metys	Research Fellow	Intern. Inst. of Cell., Belgium
	J. Roth	Medical Director	DB, NIAMDD
	M. Waelbroeck	Research Fellow	Intern. Inst. of Cell., Belgium

COOPERATING UNITS (if any)
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LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been terminated

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 47,003 - 05 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Insulin Receptors and Insulin Responsiveness in 3T3-L1 Fatty Fibroblasts.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	C Grunfeld	Research Associate	DB, NIAMDD
	E. Van Obberghen	Visiting Associate	DB, NIAMDD
	C. R. Kahn	Senior Surgeon	DB, NIAMDD
Other:	K. Baird	Biologist	DB, NIAMDD
	J. Carpentier	Charge de Recherche	
	L. Orci	Professor of Medicine	

COOPERATING UNITS (if any)
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University of Geneva
Geneva, Switzerland

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.7	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin receptors and responsiveness have been studied in undifferentiated and fatty 3T3-L1 fibroblasts. With differentiation, 3T3-L1 fibroblasts develop lipoprotein lipase. Insulin and insulin receptor antibody stimulate release of lipase from the cells as well as increases intracellular lipoprotein lipase activity. Glucocorticoid hormones induce insulin resistance in 3T3-L1 cells. The affinity of the receptor for insulin is decreased and there is inhibition of the insulin response at a post-receptor site. With differentiation, 3T3-L1 cells develop surface markings characteristic of fat cells. Insulin is taken up into the cell by a pathway utilizing coated pits.

PHS-6040

Project Number: Z01 AM 47,003 - 05 DB

Project Description:

3T3-L1 fibroblasts differentiate into a fatty phenotype which has insulin stimulated glucose transport, glucose oxidation, lipoprotein lipase and fatty acid and triglyceride synthesis, as well as insulin inhibited lipolysis. The use of an insulin-responsive cultured cell line has allowed us to explore the effects of antibody against the insulin receptor on a long-term insulin action, stimulation of lipoprotein lipase activity and to develop an in vitro model for the insulin resistance seen in patients with glucocorticoid excess. The cell line has also enabled us to study morphometrically the interaction of insulin with fat cells and to study the development of membrane invaginations characteristic of fat cells.

With differentiation, 3T3-L1 cells develop lipoprotein lipase, the enzyme responsible for cleaving external triglyceride to permit uptake of fatty acids. Insulin stimulates both the acute release of lipase from the cells and an increase in intracellular lipoprotein lipase activity. The latter requires both RNA and protein synthesis. Antibody against the insulin receptor also mimics this long-term insulin effect. The dose response curve for antibody is identical to that found for both inhibition of insulin binding and for the acute insulin-mimetic activity of the antibody. The onset of antibody stimulation of lipoprotein lipase activity is similar to that of insulin but is decreasingly effective between 12 and 24 hours. Macromolecular synthesis (and presumably gene expression) is also required for activity of the antibody.

This study has several implications for the mechanism of insulin action. Since the antibody against the insulin receptor does not interact with the insulin binding sites on the nucleus, it is unlikely that such sites are involved in this action of insulin, which does require participation of the nucleus. Likewise, it has been proposed that uptake of insulin into the cell followed by activation and partial degradation may be required for insulin's long-term effects. It is unlikely that the antibody could be processed in a similar way. Thus, we conclude that the interaction of a ligand with the insulin receptor on the cell surface with generation of a second messenger accounts for the action of insulin, or that the receptor, once activated by ligand, contains all of the necessary information for hormone action.

Glucocorticoid hormone excess causes insulin resistance in man and animals. It has not been possible to induce these changes in vitro using freshly isolated adipocytes. Exposure of 3T3-L1 cells to physiologic and pharmacologic concentrations of glucocorticoid hormones for 2-4 days produces insulin resistance. The affinity of the receptor for insulin is reduced throughout occupancy. Maximal insulin stimulation of glucose uptake is dramatically reduced. Basal uptake is slightly reduced. The cells are also less sensitive to insulin; half-maximal stimulation occurs at a 10-fold higher concentration of insulin. Since the affinity of the receptor is shifted only three-fold, this provides evidence that glucocorticoid hormones exert their effects at post-receptor steps as well as affecting the insulin receptor.

The morphology of 3T3-L1 cells, including the interaction of insulin with the cells was studied by electron microscopy. The external surface of the plasma membrane of adipocytes has many invaginations when viewed by freeze fracture techniques. When undifferentiated 3T3-L1 cells are studied by the technique, they show few invaginations. With differentiation, the numbers of invaginations increase with time, paralleling the increased responsiveness to insulin and other hormones. These studies more firmly establish the increased surface invaginations as characteristics of adipocytes and may link their presence to hormonal responsiveness.

As with other cultured cells, insulin is taken up into 3T3-L1 cells by a receptor mediated endocytosis. Using EM autoradiography we have demonstrated that binding to the plasma membrane is followed rapidly by preferential association to coated plasma membrane vesicles (coated pits). This is the first demonstration of the role of coated pits for insulin uptake in a hormonally responsive cell lines.

We have also studied the uptake of colloidal gold-albumin as a marker for the uptake of fatty acid associated with albumin. As controls we studied the uptake of cationized ferritin, an ionized ferritin and horseradish peroxidase. The elucidation of the unique method of uptake of albumin will be useful in understanding the functioning of the adipocyte.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 47,005 - 08 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of Insulin Binding in Circulating Cells of Man.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	I.G. Fantus	Visiting Fellow	DB, NIAMDD
	R.F. Dons	Clinical Assoc.	DB, NIAMDD
	G. Saviolakis	Staff Fellow	DB, NIAMDD
	G. Grunberger	Clinical Assoc.	DB, NIAMDD
	J. Ryan	Bio Lab Tech	DB, NIAMDD
	C.M. Hendricks	Bio Lab Tech	DB, NIAMDD
	P. Gorden	Medical Director	DB, NIAMDD
Others:	Laurence M. Corash	Senior Surgeon	CP, CC
	M. Cornblath	Medical Director	IRP, NICHD

COOPERATING UNITS (if any)
Intramural Research, Division of Pediatrics, and Neonatal and Pediatric Medicine Branch, National Institute of Child Health and Human Development. Hematology Service, Clinical Pathology Branch, Clinical Center

LAB/BRANCH
Diabetes Branch

SECTION
Clinical and Cellular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.3	3.3	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Numerous studies from this laboratory have investigated the interaction of insulin with circulating mononuclear cells. It has been demonstrated that obese diabetic individuals have a deficiency in insulin receptors and these are reversed by appropriate diet. Further, numerous other diseases of receptor deficiency have been identified. The present study involves insulin dependent diabetes mellitus and glucocorticoid excess states as well as an attempt to characterize the insulin receptor of the human erythrocyte.

PHS-6040

Project Number: Z01 AM 47,005 - 08 DB

Project Description:

We have studied a group of patients with insulin-dependent diabetes mellitus and have observed that insulin binding is either normal or slightly elevated. This is consistent with the severe hypoinsulinemia seen in these individuals and consistent with the animal models of insulin dependent diabetes. In addition, conditions which are seen in patients with diabetes were simulated in vitro, i.e. ketoacidosis, hyperglycemia, and acidemia. While these factors may influence binding in the patient they are not perpetuated into the in vitro assay; thus there is no "memory" for these abnormalities when taken from the patient and studied in vitro. By contrast, starvation and hyperinsulinemia are conditions for which the receptor has "memory" and the abnormalities are perpetuated into the in vitro assay. Corticosteroids in vitro increased insulin binding but in normal individuals who also develop hyperinsulinemia in response to corticosteroids we have been unable to demonstrate this effect. In a series of studies it has been shown that corticosteroids definitely influence insulin binding through the corticosteroid receptor on cultured human lymphocytes.

In a separate series of studies we have shown that human erythrocytes have specific insulin receptors and that the concentration of these receptors on the cell surface is exponentially related to cell age. Thus, red blood cells lose receptors as an expediential function of their cell age and this is independent of the reticulocyte content of the preparation. This may be the first example of an age related integral protein of the red blood cell membrane.

Insulin binding to monocytes or erythrocytes was measured in 11 patients with chronic glucocorticoid excess of diverse etiology. With monocytes, tracer ^{125}I-insulin binding was increased due to an increase in receptor affinity; a smaller, variable effect from changes in receptor concentration was noted. With erythrocytes an increase in receptor affinity was associated with a decrease in receptor concentration so that the binding of a tracer of ^{125}I-insulin was normal, but decreased at higher concentrations of the hormone. The disparity of results between these two cell types and between the present studies and previous studies suggest that the effects of glucocortocoid excess on the insulin receptor are extremely complex and that in this condition extrapolations from circulating cells to liver and muscle are of uncertain validity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,006 - 04 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Study of Insulin Receptors In Vivo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	H. Wachslicht-Rodbard	Visiting Associate	DB, NIAMDD
Other:	J. Roth	Medical Director	DB, NIAMDD
	M. Berman	Physicist	LPP, NCI

COOPERATING UNITS (if any)
Laboratory of Theoretical Biology, National Cancer Institute

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project has been inactivated

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,007 - 05 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Antibodies to Receptors: Detection in Disease States and Use as Probes to Study Receptor Function and Structure.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	L.C. Harrison	Visiting Scientist	DB, NIAMDD
	G. Saviolakis	Research Associate	DB, NIAMDD
	K. Melez	Clinical Associate	NMD, NICHHD
Other:	A. Itin	Grant Supported Tech Assistant	DB, NIAMDD
	L. Underhill	Chemist	DB, NIAMDD
	C.J. Venter	Assistant Professor	SUNY, Buffalo

COOPERATING UNITS (if any)
Neonatal Medicine Branch, National Institute of Child Health and Human Development, Department of Pharmacology, SUNY, Buffalo.

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	1.8	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Receptor antibodies have been used in a new radioimmunoassay for insulin receptors, which is being applied to study receptor phylogeny and function. A fraction of insulin non-dissociable from cells after binding has been shown to be covalently coupled to insulin receptors. Immunoprecipitation of receptors has been used as an assay to screen for receptor antibodies. Low titer IgM antibodies have been found in patients with diabetes and in the New Zealand obese (NZO) mouse - a model of human adult-type diabetes. The NZO mice also have evidence of generalized autoimmunity with antibodies to DNA and dense deposits of IgM in the renal golmeruli. Their renal pathology resembles that of both systemic lupus erythematosis and diabetes mellitus.

Project Number: Z01 AM 47,007 - 05 DB

Project Description:

The interaction of insulin receptor antibodies with the solubilized insulin receptor has been studied in detail. Solubilized insulin receptors were prepared by extracting human placental membranes with Triton X-100, and assaying for ^{125}I-insulin binding activity using polyethylene glycol to precipitate receptors. These solubilized receptors retain all the properties, including negative cooperativity, of membrane-bound receptors. Receptor antibodies inhibited insulin binding to the solubilized receptors with the same titers as previously reported using whole cells. This inhibition was due to a decrease in the number of available insulin binding sites rather than a decrease in their affinity as seen with membrane-bound receptors.

Non-Reversible Binding of Insulin:

The binding of insulin is not a totally reversible process. A large proportion of non-dissociable (internalized) insulin in cells can be shown to be complexed with receptors. The resistance of these complexes of strong denaturants such as 2% SDS or 6M guanidine HCl indicates that a fraction of the bound insulin may become covalently linked to its receptor. This is the first direct evidence that hormones "processed" by the cell are associated with receptors. However the significance of covalent insulin-receptor complexes remains to be elucidated.

Radioimmunoassay of the Insulin Receptor:

The specificity of the antibodies for precipitating the receptor has made them a useful reagent for measurement of the receptor in a classic radioimmunoassay procedure. Receptor is labeled with ^{125}I-insulin and incubated together with a fixed amount of antibody and increasing amounts of unlabeled receptor. Bound and free labeled receptor were separated by addition of a second antibody. Addition of a minimum of 10^{-10}M unlabeled insulin binding sites results in a decrease in the bound labeled receptor. This assay enables us, for the first time, to measure the receptor independent of its ability to bind insulin. We have demonstrated that receptors from different tissues and species are immunologically different and are applying the radioimmunoassay to the study of receptor ontogeny and phylogeny.

Production of Monoclonal Antibodies to the Insulin Receptor:

Partially-purified human placental insulin receptors were used to immunize Balb/C mice. After 4 days the mice spleen cells were hybridized to the P3 x 63 line of mouse myeloma cells (kindly donated by Dr. G. Eisenbarth). Several hybridoma lines grew out which appeared by several criteria to be secreting receptor antibodies. These have been passaged as ascites tumors in Balb/C mice prior to cloning.

Receptor Autoimmunity:

NZO mice sera were found to contain low titer (1/4 to 1/32) IgM antibodies to the insulin receptor, which inhibited insulin binding and immunoprecipitated the receptor. The pathogenetic role of these antibodies in the insulin resistant diabetes of these obese mice is being investigated.

These animals also develop renal lesions resembling those seen in both systemic lupus erythematosis and diabetes, and associated with the deposition of IgM on the glomerular basement membrane. Further studies will be performed in NZO mice to elucidate the role of animal factors in the pathogenesis of diabetic complications.

In conjunction with Dr. C. J. Venter of Buffalo, N. Y., we have extended the concept of receptor autoimmunity to include autoantibodies to the beta-adrenergic receptor in some patients with asthma and allergic disorders. We are currently engaged in studies to determine the prevalence, pathogenetic significance, and mechanism of action of these antibodies.

Publications:

1. Kahn, C. R., Flier, J. S., Muggeo, M., and Harrison, L. C.: Autoantibodies to the insulin receptor in insulin resistant diabetes. in The Immunology of Diabetes (W. J. Irvine, Ed), Macmillan, London, 1979.

2. Harrison, L. C., and Kahn, C. R.: Autoantibodies to the insulin receptor: Clinical significance and experimental applications. Prog. Clin. Immuno. 4: 104-125, 1980.

3. Harrison, L. C., and Roth, J.: The pathophysiology of insulin receptors. A model for peptide hormone action. Aust. New Zeal. J. Med. 10: 78-88, 1980.

4. Harrison, L. C., Van Obberghen, E., Grunfeld, C., King, G. L., and Kahn, C. R.: Modulation of the insulin receptor by insulin receptor autoantibodies. in Cell Membranes, Receptors, and the Immune Response (R. Cohen and H. Kohler, eds), Alan Liss, Inc., New York, pp. 109-126, 1980.

5. Venter, J. C., Fraser, C. M., and Harrison, L. C.: Autoantibodies to the β_2-adrenergic receptor: A possible cause of β-adrenergic hyporesponsiveness in allergic rhinitis and asthma. Science 207: 1361-1363, 1980.

6. Harrison, L. C.: Insulin receptor autoantibodies: Clinical and molecular aspects. Circulation Research, in press.

7. Harrison, L. C., and Flier, J. S.: Diabetes associated with other endocrine diseases. in Secondary Diabetes: The Spectrum of the Diabetic Syndrome (S. Podolsky and M. Viswanahan, eds), Raven Press, New York, in press.

8. Melez, K., Harrison, L. C., Gilliam, J. N., and Steinberg, A. D.: Diabetes is associated with autoimmunigy in the New Zealand obese (NZO) mouse. Diabetes, in press.

9. Kahn, C. R., Harrison, L. C., Flier, J. S., Muggeo, M., Van Obberghen, E., Grunfeld, C., Karlsson, F. A., King, G. L., and Roth, J.: Antibodies to the insulin receptor and insulin-resistant diabetes. in Autoimmune Aspects of Endocrine Disease (A. Pinchera, ed), Academic Press, London, in press.

Awards:

A.I. is supported by a grant from the Juvenile Diabetes Foundation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,009 - 04 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Anti-receptor Antibodies as Probes of Insulin Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	C.R. Kahn	Senior Surgeon	DB, NIAMDD
	G.L. King	Research Associate	DB, NIAMDD
	K.L. Baird	Bio Lab Tech	DB, NIAMDD
Other:	M.M. Rechler	Senior Surgeon	LBP, NIAMDD
	S.P. Nissley	Senior Investigator	MET, NCI
	J. Larner	Prof of Biochemistry	Univ of Va., Charlottesville

COOPERATING UNITS (if any)
Laboratory of Biochemical Pathology, NIAMDD; Metabolism Branch, National Cancer Institute; Department of Biochemistry, Univ. of Va., Charlottesville, Virginia

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 47,010 - 08 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Patient studies using MSA (Multiplication stimulating activity) Radioreceptorassay.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.M. Hendricks	Bio Lab Tech	DB,NIAMDD
	P. Gorden	Medical Director	DB,NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION
Clinical and Cellular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

MSA (Multiplication stimulating activity) is an insulin-like peptide which reacts with its own receptor and the insulin receptor. MSA is elevated in about 40% of patients with hypoglycemia and non-islet cell tumors. Patients with hemangiopericytoma have elevated circulating MSA levels.

PHS-6040

Project Number: Z01 AM 47,010 - 08 DB

Project Description:

This study was originally designed to study the physiology and pathophysiology of circulating growth factors in man. When a crude NSILA-s preparation or a highly purified multiplication stimulating activity preparation was incubated with purified liver membranes, a highly sensitive assay for these growth peptides was developed. The most recent aspect of this study is an attempt to characterize those patients who are most likely to demonstrate an elevation of an MSA cross-reacting material. Thus, in a study of over 52 patients with tumor-induced hypoglycemia of a non-insulin nature we have found that 6 of 7 patients with hemangiopericytoma have elevations of the circulating growth factor-like material. Since these materials are insulin-like and since in a single patient we have shown that the level falls as the patient goes into remission, we feel that this circulating insulin-like material is, at least in part, responsible for the hypoglycemia seen in these patients. Further studies are in progress to define further the tumor types most typically related to an elevation of the non-suppressable insulin-like activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,016 - 04 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Structure of the Insulin Receptor Studied by Ionizing Radiation.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	J.T. Harmon	Guest Worder	DB, NIAMDD
	C.R. Kahn	Senior Surgeon	DB, NIAMDD
Other:	E.S. Kempner	Senior Investigator	LPB, NIAMDD
	W. Schlegel	Visiting Fellow	LNE, NIAMDD
	M.L. Johnson	Assis. Professor	

COOPERATING UNITS (if any)
Laboratory of Physical Biology, NIAMDD
Laboratory of Nutrition and Endocrinology, NIAMDD
Diabetes Center, Univ. of Virginia, Charlottesville, VA

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The components of the insulin receptor have been examined using SDS polyacrylamide gel electrophoresis for the IM-9 lymphocyte receptor and using radiation inactivation for the rat liver membrane receptor. The results from radiation inactivation studies suggest that the insulin receptor is composed of at least two components. One component appears to be a high molecular weight affinity-regulator of a second component the insulin binding component, which appears to have a lower molecular weight. Computer analysis of these results indicate a possible model for the insulin receptor would be a polymer of binding components plus affinity-regulatory components.

PHS-6040

Project Number: Z01 AM 47,016 - 04 DB

Project Description:

We have previously shown that high energy ionizing radiation can be used to determine the molecular weight of the insulin receptor in situ in rat liver membranes. When the log of the fraction of specific insulin binding was plotted versus radiation dose, complex results were observed. Binding increased 2-3 fold reaching a maximum on membranes exposed to 12 Mrad and then decreased at higher doses of radiation. Similar results for insulin binding have been observed with adipocyte membranes, IM-9 lymphocyte membranes and Triton X-100 solubilized rat liver membranes. Binding of epidermal growth factor and multiplication stimulating actively did not increase with irradiation of rat liver membranes. From equilibrium studies, it was concluded that the increase in binding at low radiation doses was due to an increase in binding affinity. The total number of receptors decreased at all radiation doses. These data suggest that at least two membrane components are involved in the insulin receptor interaction: one with an apparent molecular weight of 300,000-350,000 which appears to regulate the affinity of the insulin binding protein and is inactivated at low doses of radiation; and a second component with molecular weight of 90,000-100,000 which binds the insulin. In the intact membranes the effect of the affinity-regulator is to lower the affinity of the insulin binding component for insulin. Insulin on the receptor at the time of the irradiation appears to reduce the interaction between the binding and affinity-regulatory components. Computer analysis of the results of radiation inactivation studies are most consistent with a model in which there is a separate affinity-regulator associated with the insulin receptor. However, this simple model will not predict enhanced dissociation of ^{125}I-insulin caused by an excess of unlabeled insulin (negative cooperative interactions). Thus a proposed model of the insulin receptor in the membrane would be a polymer of the binding plus affinity regulator.

Publications:

1. Lang, U., Kahn, C.R., and Chrambach, A.: Characterization of the insulin receptor from human lymphocytes by quantitative polyacrylamide gel electrophoresis. Endocrinol. (1980), 106, 40-49.

2. Lang, U., Kahn, C.R., and Harrison, L.C.: Subunit structure of the insulin receptor in the human lymphocyte. Biochemistry (1980), 19, 64-70.

3. Harmon, J.T., Kahn, C.R., Kempner, E.S. and Schlegel, W.: Characterization of the insulin receptor in its membrane environment by radiation inactivation. J. Biol. Chem. (1980) 255, 3412-3419.

Awards:
JTH is recipient of the 1980-81 Solomon A Berson Research and Development Award of the American Diabetes Association, Inc.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,018 - 03 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cellular Insulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	J. Rosenzweig	Clinical Associate	DB, NIAMDD
	M.A. Lesniak	Research Chemist	DB, NIAMDD
	J. Roth	Medical Director	DB, NIAMDD
	B. Samuels	Research Assistant	DB, NIAMDD
	D. LeRoith	Visiting Scientist	DB, NIAMDD
	R. Pruss	Staff Fellow	DB, NIAMDD
	F. de Pablo	Visiting Fellow	DB, NIAMDD
Other:	M.S. Brownstein	Medical Officer	LCS, NIMH

COOPERATING UNITS (if any)

Laboratory of Clinical Medicine, National Institute of Mental Health

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have found in the guinea pig a second insulin which is natural to the extrapancreatic tissues and is not found in plasma. This insulin has biological activity and immunoreactivity similar to typical mammalian insulins, in marked constrast to the bizarre secretory insulin found in the guinea pig pancreas. These data provide evidence for the extrapancreatic synthesis of insulin and provide new explanations for the mystery of insulin evaluation in the hystricomorph rodents. A substance extracted from drosophila and earthworms is very similar to mammal insulins.

Project Number: Z01 AM 47,018 - 03 DB

Project Description:

In previous studies, we have demonstrated the presence of insulin in brain, liver, and essentially all other tissues of rats and humans in levels multifold higher than plasma. Tissue insulin was indistinguishable from genuine insulin by radioimmunoassay, gel filtration, bioactivity in fat cells, and neutralization of activity with anti-insulin antibodies.

Insulin was also found in human IM-9 cultured lymphocytes and in cultured human fibroblasts in concentrations simillar to cells in vivo, and multifold higher than the concentration of insulin in fetal calf serum from the media. The cultured human cell insulin would not bind to a monoclonal anti-beef insulin antibody, suggesting that it is human insulin synthesized by the cells rather than beef insulin taken up from the media.

These experiments suggest that extrapancreatic tissues synthesize insulin. In more recent studies, we have found that guinea pigs have two insulins--one synthesized and secreted by the pancreas into plasma and a second insulin native to the extrapancreatic tissues.

The insulin secreted by the β cells of the guinea pig islets differ radically in structure from all other mammalian insulins so that its biopotency and receptor binding activity is only 2% that of all other mammalian insulins, even in the guinea pig. By the use of a standard radioimmunoassay for pork insulin and other typical mammalian insulins, insulin immunoreactivity was found in acid-ethanol extracts of liver, brain, and other extrapancreatic tissues of the guinea pig at concentrations similar to those found previously in extrapancreatic tissues of rat, mouse and humans. The insulin-like immunoreactivety eluted from G-50 Sephadex in two peaks; the major peak co-eluted in regions of insulin marker, and pooled material from this peak had bioactivity (glucose oxidation in isolated fat cells) appropriate for its pork-like insulin immunoreactivity. Significantly, the bioactivity was largely neutralized by anti-beef and anti-pork insulin antibodies but not by anti-guinea pig insulin antibodies. While all tissues had measurable amounts of this material, none was detected in plasma.

Using another radioimmunoassay that is specific for guinea pig (pancreatic) insulin, we detected typical guinea pig insulin in the pancreas, plasma, liver, and kidney, but little or none in extracts of other tissues. As expected, the guinea pig insulin in these extracts had reduced bioactivity compared with most mammalian insulins, and the bioactivity was neutralized by anti-guinea pig but not anti-beef insulin antibodies.

Therefore, the guinea pig has at least two sets of insulin genes: The newly discovered one that codes for a typical mammalian insulin that is found in all extrapancreatic tissues and a second one that codes for the bizarre insulin that is typically found in the β cell and blood of the guinea pig. These data (a) lend strong support to our conclusions that much of the insulin found in extrapancreatic tissues is synthesized locally rather than in the pancreas and (b) shed new light on the mystery of how the guinea pig had evolved such a bizarre insulin.

An acid-ethanol extraction of drosophilia and earthworms revealed a substance that by all criteria described above is very similar to mammalian insulin. Studies are in progress to further characterize this insulin-like substance. In addition, we are studying chick embryos, which have a pancreas but does not appear until days 5-7. We find insulin in 2-4 day chick embryos. This result supports the idea that insulin production is a property not unique to β cells of vertebrate pancreases.

Publications:

1. Rosenzweig, J.L., Havrankova, J., Lesniak, M.A., Brownstein, M., and Roth, J.: Insulin is ubiquitous in the extrapancreatic tissues of rats and humans. Proc. Natl. Acad. Sci., USA 77:572-576, 1980.

Awards:

The following awards were presented to J. Roth.

1. The First Lita Annenberg Hazen Award For Excellence in Clinical Research ($100,000).

2. Public Health Service Distinguished Service Medal.

3. Honorary Professorship at the University of Uppsala, Uppsala, Sweden.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 47,019 - 03 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Morphological Studies of Ligand Binding to Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	P. Gorden	Senior Surgeon	DB, NIAMDD
	J.-L. Carpentier	Charge de Recherches	Univ of Geneva
	E. Van Obberghen	Visiting Associate	DB, NIAMDD
	M.A. Lesniak	Research Chemist	DB, NIAMDD
	N. Hizuka	Visiting Fellow	DB, NIAMDD
Other:	L. Orci	Prof of Medicine	Univ of Geneva
	P. Freychet	Prof of Medicine	Univ of Nice
	L. Le Cam	Charge de Recherches	Univ of Nice

COOPERATING UNITS (if any)
Institute of Histology and Embryology, University of Geneva, Geneva, Switzerland; Department of Med., University of Nice, Nice, France: Dept. of Biochem.

LAB/BRANCH
Diabetes Branch

SECTION
Clinical and Cellular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	2.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

^{125}I-insulin binds to IM-9 cells (human) and hepatocytes (rat). By using quantitative EM autoradiography as a probe, it can be shown that labeled insulin is internalized by a pro ess which appears to be time and temperature-dependent. Other labeled proteins (low density lipoprotein, epidermal growth factor, human growth hormone, glucogon, and anti-insulin receptor antibody) appear to behave in the same fashion i.e. initial binding followed by internalization.

Project Number: Z01 AM 47,019 - 02 DB

Project Description:

This study was designed to visualize the interaction of a ligand with a cell from initial binding through steady-state binding. In as much as kinetic states of polypeptide hormones as well as many other ligands are done with ^{125}I-labeled material we chose EM autoradiography as our visual probe.

Our initial studies demonstrated that the labeled ligand bound to the plasma membrane at early time points of incubation and at low temperatures but with increasing temperature and time there was progressive internalization of the ligand. We have now extended these studies to insulin in cultured human and rodent hepatocytes. In addition, we have studied epidermal growth factor in human fibroblasts as well as low density lipoprotein in human fibroblasts. These studies all reveal a similar qualitative process that has initial binding followed by internalization. In all of these systems the internalized label material preferentially localizes in lysosomal structures. When the volume density of these lysosomal structures are studied quantitatively, it is found that they are increased in regions with autoradiographic grains. Thus, the binding is occurring to lysosomal structures which preferentially reside in the Golgi region of the cell.

It would appear that this is a general mechanism related to multiple different types of ligands including the above mentioned as well as anti-insulin receptor antibody, glucagon, and growth hormone and that the process may function to remove the ligand from the cell surface and cause its degradation. In addition, other studies indicate that this may also be a mechanism by which the cell surface receptor is lost.

Additional studies are in progress to evaluate the effects of inhibitors of lysosomal function on the process of hormone degradation and receptor regulation.

Publications:

1. Carpentier, J.-L., Gorden, P., Freychet, P., Le Cam, A., and Orci, L.: ^{125}I-insulin is preferentially internalized in regions of the hepatocytes rich in lysosomal and Golgi structures. Experientia, 35:904-905 1979.

2. Carpentier, J.-L., Gorden, P., Goldstein, J.L., Anderson, R.G.W., Brown, M.S., and Orci, L: Binding and internalization of ^{125}I-LDL in normal and mutant human fibroblasts: A quantitative autoradiographic study. Exp. Cell Res., 121: 135-142, 1979.

3. Gorden, P., Carpentier, J.-L., Van Obberghen, E., Barazzone, P., Roth, J., and Orci, L.: Insulin-induced receptor loss in the cultured human lymphocyte: Quantitative morphological perturbations in the cell and plasma membrane. J. Cell Sci., 39: 77-88, 1979.

4. Carpentier, J.-L., Gorden, P., Freychet, P., Le Cam, A., and Orci, L.: Relationship to binding to ^{125}I-insulin in isolated rat hepatocytes. Diabetologia 17: 379-384, 1979

5. Gorden, P., Carpentier, J.-L., Freychet, P. and Orci, L.: Internalization of polypeptide hormones: Mechanisms intracellular localization and significance. Diab. 18: 263-274, 1980

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 47,020 - 02 DB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Insulin Antibodies, Insulin Allergy, and Insulin Resistance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	C.R. Kahn	Senior Surgeon	DB, NIAMDD
Other:	A. Rosenthal	Senior Investigator	LCI, NIAID
	D. Mann	Senior Investigator	I, NCI

COOPERATING UNITS (if any)

Laboratory of Clinical Investigation, Natl. Inst. of Allergy and Infectious Dis.
Immunology Branch, National Cancer Institute

LAB/BRANCH

Diabetes Branch

SECTION

Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been terminated

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 47,021 - 02 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Insulin Receptors and Action in Cultured Human Fibroblasts.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	C.R. Kahn	Senior Surgeon	DB, NIAMDD
	J. Podskalny	Biologist	DB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.2	1.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin binding and action have been studied in cultured human fibroblasts. Cells are derived from insulin resistant patients and from diabetics for comparison to controls. Activation of glycogen synthase, stimulation of AIB transport, and incorporation of ^{3}H-thymidine into DNA are all being examined in these cells. Receptor defects have been found in cells from a patient with the Type A syndrome of insulin resistance and acanthosis nigricans and in an infant with leprechaunism.

PHS-6040

Project Number: Z01 AM 47,021 - 02 DB

Project Description:

We are examining insulin binding and insulin action in cultured cells from patients with diabetes mellitus or insulin resistant states. Cultured skin fibroblasts are an accessible tissue amenable to *in vitro* manipulations. Activation of glycogen synthase represents an "early" effect of insulin in these cells. Stimulation of the transport of d-amino isobutyric acid (AIB), a non-metabolizable amino acid, is being studied as representative of a "delayed" effect of insulin's action. Finally, ^{3}H-thymidine incorporation into DNA, occurring at high insulin concentrations, is representative of the mitogenic or "late" action of insulin.

Cells from a patient with the Type A syndrome of insulin resistance and acanthosis nigricans and also cells from an infant with leprechaunism, appear to exhibit the same basic defect in insulin binding. ^{125}I-insulin binding is decreased; there is a loss of negative cooperativity among receptors; the concentration of insulin required to achieve half-maximal displacement is increased 10-fold; anti-insulin receptor antibody does not recognize the receptors on these cells; and multiplication stimulating activity (MSA) is more potent in displacing ^{125}I-insulin bound to these insulin resistant cells than that bound to control cells. Despite these anomalies, insulin still activates glycogen synthase and stimulates AIB transport. The cells do have a diminished capacity to respond to the mitogenic action of insulin however. Other cell strains derived from patients with lipoatrophic diabetes, extremely early onset diabetes, and Type A insulin resistance are also being examined

Publications:

1. Kahn, C. Ronald, Podskalny, Judith M. Demonstration of a Primary (?Genetic) Defect in Insulin Receptors in Fibroblasts from a patient with the Syndrome of Insulin Resistance and Acanthosis Nigricans Type A. JCEM 50:1139-41, 1980

2. Moses, A.C., Nissley, S.P., Short, P.A. Rechler, M.M., Podskalny, J.M., Purification and Characterization of Multiplication Stimulating Activity Eur. J. Bioch. 103:387-400, 1980.

3. Podskalny, J.M., Kahn, C.R. Insulin Activates Glycogen Synthase in Cultured Human Fibroblasts. Diabetes, in press.

4. Rechler, M.M., Zopf, J., Nissley, S.P., Froesch, E.R., Moses, A.C., Podskalny, J.M., Schilling, E.E., Humbel, R.E. Interactions of Insulin-like Growth Factors I and II and Multiplication Stimulating Activity with Receptors and Serum Carrier Proteins. Endocrinology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,022 - 01 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Insulin Receptors in Syndromes of Extreme Insulin Resistance.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	S. I. Taylor	Research Associate	DB,NIAMDD
	J. Roth	Medical Director	DB,NIAMDD
	R. Blizzard	Guest Worker	DB,NIAMDD
	I. Spitz	Visiting Scientist	DB,NIAMDD
	A.E. Brasel	Assis. Professor	Hennepin County
	T. Pokors	Fellow	Medical Center
	R.R. Engel	Assoc. Professor	Dept. of Pediatrics
			Minneapolis, Minn.

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.8	

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

B-lymphocyte cell lines were established using Epstein-Barr virus from peripheral blood of normal volunteers, juvenile-onset diabetics, and patients with extreme insulin resistance. Cells from patients with juvenile-onset diabetes, lipoatrophic diabetes, and Type B extreme insulin resistance had normal ^{125}I-insulin binding. A patient with Type A extreme insulin resistance and a patient with leprechaunism had decreased binding of ^{125}I-insulin. This resulted from a decreased number of insulin receptors. The residual insulin receptors appeared to have normal properties.

Project Number: Z01 AM 47,022 - 01 DB

Project Description:

Extreme insulin resistance is a rare syndrome. Among the causes are antibodies to insulin, antibodies to the insulin receptor, defects in insulin binding, and defects in the steps of insulin action subsequent to hormone binding. We have concentrated primarily on genetic syndromes defects either in insulin binding or post-binding events. The wide variety of genetic syndromes of extreme insulin resistance provides a collection of mutations which may affect any of the early steps in insulin action. By creating permanent cell lines from these patients' cells we hope to obtain a set of tools which will contribute to the elucidation of the poorly understood mechanism of insulin action.

Using Epstein-Barr virus, we have established B-lymphocyte cell lines from peripheral blood of normal volunteers, as well as patients with juvenile-onset diabetes, lipoatrophic diabetes, Type A extreme insulin resistance, Type B extreme insulin resistance, and leprechaunism. With the exception of cells from a patient with Type A extreme insulin resistance all other patients' cells had markedly diminished ^{125}I-insulin binding. This resulted from a decreased number of insulin receptors. The residual receptors had normal properties as judged by specificity of hormone binding as well as ability to bind anti-receptor antibody. Studies are underway to determine whether we can identify cellular responses to insulin in the transformed B-lymphocytes. Such responses to insulin might allow us to define the nature of the defect in insulin resistant patients with normal ^{125}I-insulin binding.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,023 - 01 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Growth Promoting Effect of Insulin.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. King	Research Associate	DB, NIAMDD
	C.R. Kahn	Senior Surgeon	DB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin's ability to stimulate chronic or growth responses was investigated by testing various insulin analogues's ability to stimulate ^{3}[H] thymidine incorporation into normal human fibroblasts in culture. Using human insulin synthized from amino acids, we have demonstrated that insulin molecule itself has the ability to stimulate growth and this ability is not due to any contamination of the preparation. In addition insulin's ability to stimulate growth appears to be much more conserved than its metabolic effects.

In addition we have found evidence that in rat adipocytes, there could be interaction between insulin receptor and MSA (a growth factor) receptors.

PHS-6040

Project Number: Z01 AM 47,023 - 01 DB

Project Description:

Insulin and the insulin-like growth factors, such as MSA (Multiplication Stimulating Activity), are chemically related polypeptides that have common biological activities and cross-react with each other's specific receptors. Both insulin and MSA produce acute metabolic responses (glucose oxidation in adipocytes) and chronic growth responses (DNA synthesis in cultured fibroblasts). To determine whether these effects are mediated by insulin receptors, MSA receptors or both, we have selectively blocked insulin receptors with a pure insulin receptor antagonist, namely, Fab fragments derived from naturally-occurring antibodies to the insulin receptor. In adipocytes 10 µg/ml of Fab inhibited insulin binding by 90% whereas MSA binding was inhibited by only 5%. This blockade of insulin receptors produced a 30-fold shift in the dose response for stimulation of glucose oxidation by both insulin and MSA, suggesting that the acute metabolic effect of both peptides is mediated via the insulin receptor. In cultured human fibroblasts 10 µg/ml of Fab inhibited insulin binding by 90% and MSA binding by 15%. In this case, however, blockade of the insulin receptor did not alter the dose response for stimulation of thymidine incorporation into DNA by either insulin or MSA. Furthermore, intact anti-receptor antibody which produces multiple other insulin-like effects did not stimulate thymidine incorporation by itself. These data demonstrate directly that insulin and MSA mediate their metabolic effects via interaction with the insulin receptor, whereas the growth-promoting action of both peptides is mediated via the receptor for MSA and other growth factors.

Insulin and insulin-like growth factors (IGF) are polypeptide hormones that share many common biological activities. Binding data showed that these hormones bind to their own receptors with high affinities but can bind to each other's receptors with low affinities. By using a competitive inhibitor of insulin receptor, the monovalent fragment of anti-insulin receptor antibody, we have demonstrated that insulin and Multiplication Stimulating Activity (MSA, and an IGF) are mediating their common metabolic activities such as glucose oxidation through the insulin receptor. Their growth effects such as thymidine incorporation, on the other hand, are mediated through a growth receptor.

To extend this study, we have proceeded to characterized insulin's ability to stimulate growth. We have compared >25 different analogues of insulin in their ability to stimulate both the conversion of ^{14}C-glucose $\rightarrow {}^{14}CO_2$ in adipocytes and the incorporation of ^{3}[H] thymidine to DNA in human fibroblasts. Using pork insulin as the standard, a relative order of potency could by defined for each biological activity. Insulin used in the study included naturally occurring and chemically modified insulins.

Data from these comparisons showed that the relative potencies of insulin's ability to stimulate growth are completely different from insulin's effect on metabolic or acute activities. Insulins from the Hystricomorph family such as casiragua, porcupine and guinea pig showed that greatest difference between the two biological activities. In addition casiragua and porcupine insulins were able to stimulate thymidine incorporation into human fibroblast to a greater level than pork insulin. From these data we have demonstrated that insulin molecule itself has the ability to stimulate growth. This effect resides in the insulin molecule and is not due to con-

tamination in the insulin preparation since human insulin synthized from amino-acids has the same potency as pork insulin. Secondly insulin's ability to stimulate growth appears to be more conserved phylogenetically than its metabolic effects. Certain aspects of the insulin molecules appear to be more important for the metabolic activation than for growth effects.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,024 - 01 DB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biosynthetic Labeling of the Insulin Receptor.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. Van Obberghen	Guest Worker	DB, NIAMDD
	M. Kasuga	Guest Worker	DB, NIAMDD
	L.C. Harrison	Visiting Scientist	DB, NIAMDD
	J.A. Hedo	Guest Worker	DB, NIAMDD
Other:	A. Le Cam	Guest Worker	LMB, NCI
	A. Itin	Guest Worker	DB, NIAMDD

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, NCI

LAB/BRANCH
Diabetes Branch

SECTION Cellular and Molecular Physiology Section
Receptors and Hormone Action

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	2.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The insulin receptor of cultured human lymphocytes has been biosynthetically labeled. The ^{35}S-receptor has subunits with molecular weights of 130,000 and 94,000. The subunits of the ^{35}S-receptor are similar to those of the insulin binding site covalently labeled with ^{125}I-insulin. Lectins are being used as tools to prove receptor function and to purify receptor.

Project Number: Z01 AM 47,024 - 01 DB

Project Description:

In this study we have identified the insulin receptor of the human lymphocyte by biosynthetic labeling with ^{35}S-methionine and precipitation with antibodies against the insulin receptor. IM-9 lymphoctyes were cultured with ^{35}S-methionine, and extracted with Triton X-100. Insulin receptors were concentrated and purified 20-fold by chromatography of the extract on wheat germ agglutinin-agarose. Receptors were then precipitated quantitatively and specifically by anti-receptor serum, after addition of second antibody or Protein A-Sepharose. Recovery of ^{35}S-activity was 0.01%. Analysis by sodium dodecylsulfate-polyacrylamide gel electrophoresis under reducing conditions followed by auto-radiography revealed specific precipitation of a major band of molecular weight 94K and a minor 130K band. Bands of the same molecular weight were also seen with ^{125}I-insulin covalently cross-linked by disuccinimidyl suberate to lymphocyte plasma membranes, before and after immunoprecipitation with anti-receptor IgG. With external labeling, however, the 130K band was predominant.

Glycosylation seems to be a common property of most, if not all, cell surface proteins. Lectins bind to the carbohydrate moieties of glycoproteins and are, therefore, useful cell membrane probes. The aim of our work was to study systematically the interaction of the solubilized insulin receptor from two different human tissues, placenta and IM-9 cultured lymphocytes with a panel of twelve immobilized lectins. Six lectins (concanavalin A, lentil, pea, ricin I, ricin II and wheat germ) bound the receptor and resulted in its significant purification. The appropriate combination of several lectins with different specificities should increase the purification of an individual glycoprotein and the insulin receptor subjected to affinity chromatography in sequence of different lectin columns. However, sequential lectin chromatography did not result in further purification of either placental or lymphocyte receptor. A similar result was obtained with receptors for multiplication stimulating activity (MSA) and epidermal growth factor (EGF). The potential usefulness of sequential lectin chromatography appears to be limited by the fact that a number of membrane glycoproteins, including the insulin receptor, have several common carbohydrate determinants. These common saccharide specificities appear to be neither receptor nor tissue specific.

An additional finding of this work was that lectin affinity chromatography on these six lectins resulted in a permanent increase in the affinity of the eluted insulin receptor. This affinity change was progressive on sequential lectin chromatography. Thus, our data suggest the existence of a regulator of the insulin receptor that normally inhibits the affinity of the binding site and is separated from it by chromatography on lectins.

ANNUAL REPORT OF THE PEDIATRIC METABOLISM BRANCH
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

In the fiscal year 1979-80 clinical and laboratory investigations related to the inherited generalized disease, cystic fibrosis, have continued.

I. POLYAMINE STUDIES

The diamine putrescine and the polyamines spermidine and spermine are important compounds in the early stages of growth. Abberrant circulating, urinary and/or tissue levels of these ubiquitous aliphatic amines have been reportedin a number of diseases including: cystic fibrosis, many types of cancer, psoriasis, systemic lupus erythematosus, and hereditary muscular dystrophies. A greater understanding of the biochemistry and physiology of these metabolites will increase our knowledge of various processes involved in growth and may enhance our understanding of the underlying basis of one or more of the above mentioned diseases.

2-Pyrrolidone Pathway:

The enzymatic steps in spermidine and spermine formation from putrescine have been studied in detail and numerous studies have been carried out attempting to elucidate the manner in which their synthesis is regulated. Considerably less, however, is understood with respect to the metabolic steps, distribution, function and regulation of alternate metabolic pathways from putrescine.

Initial studies revealed that the major product of putrescine metabolism found in spent medium from sliced rat liver is 2-pyrrolidone, a lactam shown for the first time to be synthesized in any biological system (Lundgren, D.W. and Hankins, J. (1978) J. Biol. Chem. 253, 7130-7133). Concurrent with these studies was the report that 2-pyrrolidone is a normal constituent of rat brain. Earlier it was shown that Pseudomonas aeruginosa can utilize 2-pyrrolidone as its sole carbon and nitrogen source. It was of interest, therefore, to determine if in mammalian systems 2-pyrrolidone is a metabolic end product or an intermediate in an alternate metabolic pathway.

To answer the above question, 2-[^{14}C] pyrrolidone was chemically synthesized from [^{14}C] γ-aminobutyric acid and incubated with sliced rat liver in a defined medium. Chromatography of the spent medium revealed that sliced rat liver readily converts 2-pyrrolidone to at least one product.

The unknown 2-[^{14}C] pyrrolidone metabolite was partially purified by chloroform/methanol extraction, activated charcoal chromatography, and two-dimensional thin layer chromatography on silica gel plates. The 2-pyrrolidone metabolite was derivatized with bis(trimethyl) trifluroacetamide and analyzed by gas-chromatography and mass spectrometry. The mass of the molecular ion (245) and fragment ions suggested that the unknown was 5-hydroxy-2-pyrrolidone. Chemically synthesized 5-hydroxy-2-pyrrolidone had an identical mass spectrum and co-chromatographed on silica gel sheets with the unknown obtained directly from incubation medium. This is the first

demonstration of the biosynthesis of 5-hydroxy-2-pyrrolidone in any biological system.

Several effector compounds, but not necessarily the same ones were shown to inhibit or enhance, or both, the conversion of putrescine to 2-pyrrolidone and 2-pyrrolidone to 5-hydroxy-2-pyrrolidone.

Studies are presently being carried out to partially purify and characterize the enzymes in this new metabolic pathway.

It is of interest to note that unlike putrescine, spermidine and spermine which are strong cations at physiological pH, 2-pyrrolidone and 5-hydroxy-2-pyrrolidone carry no net charge (Dr. David Lundgren, NIAMDD).

II. THE AUTONOMIC NERVOUS SYSTEM IN CYSTIC FIBROSIS

A series of physiologic and biochemical studies performed in this Branch in collaboration with others (Davis, PB, Laundon, SCH. J. Lab. Clin. Med. 96:75-85, 1980; Davis, PB. J.R.L.S. Med. Sci. 8:413, 1980; Lake, CR, Davis, PB et al. Clin. Chem. Acta 92:141-146. 1979; Kaliner, M, Shelhamer, J, Smith,L, Davis, PB, and Venter, J. Am. Int. Med., (in press); Davis, PB, Braunstein, M, and Jay, C. Ped. Res. 12:703-707, 1978; Davis, PB, Hill, SC, and Ulane, M. Ped. Res. (in press); Davis, PB et al. New Engl. J. Med. 302:1453, 1980) suggest that the phenotype and genotype of CF is marked by a constellation of autonomic nervous system abnormalities which include α-adrenergic and cholinergic hyperresponsiveness and β-adrenergic resistence, and that these abnormalities might contribute to the physiopathology of CF and indicate a more general membrane dysfunction.

Investigation of the fatty acid composition of lipid in CF membranes:

To check the possibility of altered fatty acid composition contributing to the observations of decreased responsiveness of leukocytes to stimulation by isoproterenol as measured by the formation of cyclic AMP, analyses of the fatty acid composition of monocyte phospholipids were performed.

Recently Hirata et al (PNAS 75:1718, 1978, PNAS 76:368, 1979) have shown that in the erythrocyte, reticulocyte and other cells, there is a transmethylation pathway present in plasma membrane and catalyzed by two enzymes. If there is a defect in this pathway, an uncoupling phenomenon occurs between the stimulation of the β receptor in the formation of the cyclic AMP and there is an increased concentration of the substrates phosphatidylethanolamine and phosphatidylserine on the inner side of the plasma membrane and a decreased biosynthesis of phosphatidylcholine on the outer membrane. In this case, total arachidonic acid in the phospholipids is increased since it is the predominant fatty acid in the C-2 position of these phospholipids.

Preliminary experiments in 4 CF patients with pancreatic insuffiency and 1 CF patient without pancreatic insufficiency have shown an increased percentage of arachidonic acid and thus suggest that there may be a defect

in the transmethylation of phospholipid in leukocytes in this disorder. The fact that this alteration in the percentage of arachidonic acid is found in CF patients regardless of the presence or absence of pancreatic insufficiency and therefore cannot be attributed to malabsorption makes this observation more intriguing. Such studies are being continued on additional CF patients with and without pancreatic insufficiency and on obligate CF heterozygotes. (Dr. Van S. Hubbard, NIAMDD; Dr. F. Hirata, NCS, NIMH).

III. CLINICAL STUDIES IN CYSTIC FIBROSIS

1. A survey of the energy and nutritional intake of patients with CF:

Although generally it is thought that the CF patient has a voracious appetite and ingests large quantities of food, it is becoming apparent that most CF patients do not fit this description. A written record of all food and beverages consumed over a period of time was obtained from our clinic population. Out of the 24 responses received to date, only 2 patients were consuming 150% or greater of their suggested recommended dietary allowance. Of the remaining 22 patients, 10 (Group A) had intakes of less than 100% of the recommended dietary allowance and 12 (Group B) had intakes between 101 and 150% of the recommended dietary allowance. All of these patients had values for serum vitamin A, vitamin E, calcium, magnesium and (when measured) zinc in the normal range. In our study population, total energy intake did not correlate with either height or weight. Breakdown of the total energy intake into percent contributed by protein, carbohydrate and fat was similar in all groups and in the same proportion as recommended by the United States Dietary Guidelines. Means and ranges for Groups A and B have been tabulated as follows.

Anthropometric Measurements and nutrient intake in patients with cystic fibrosis

Measurements	Group A n=10	Group B n=12
Height percentile for age	38(1-75)	35(1-97)
Weight percentile for age	30(1-79)	16(1-55)
Total energy intake (as % of RDA for age)	80(52-93)	115(102-135)
Protein intake (as % of RDA for age)	193(125-279)	204(125-250)
Protein intake (as % of total energy)	18(14-26)	14(8-19)
Carbohydrate intake (as % of total energy)	50(38-63)	53(44-79)
Fat intake (as % of total energy)	33(22-45)	32(14-40)

(Dr. Van Hubbard, NIAMDD; Pamela Mangrum, CC, Nutr.)

2. The use of cimetidine to improve the malabsorption in patients with CF and pancreatic insufficiency:

As an adjunct to pancreatic enzyme supplements in CF patients with exocrine pancreatic insufficiency a 6-week period of treatment of cimetidine was tried. The rationale was that cimetidine, and H_2-receptor antagonist, would decrease gastric acid output and thereby limit gastric inactivation of the supplemental enzymes. In 8 patients studied so far steatorrhea and

azotorrhea improved and all had a significant weight gain. No changes were noted in other clinical laboratory parameters and specifically in the plasma lipid fatty acid composition.

However, in a non-CF patient, with pancreatic insufficiency as a result of total pancreatectomy, whose fatty acid composition of plasma lipids originally was almost identical to that observed in CF patients was completely normalized after prolonged cimetidine treatment. Based on this observation as well as the favorable results observed in CF patients on a short term use of this drug, a one year clinical trial utilizing cimetidine as an adjunct to pancreatic enzyme supplement has been initiated. It is interesting in this respect that, in addition to the objective changes mentioned, the patients themselves felt so much improved that they have requested continuation of cimetidine for a longer period of time. (Dr. Van S. Hubbard, Dr. Lucille Lester, Dr. Alan Egge, Dr. Paul A. di Sant'Agnese, NIAMDD).

3. Evaluation of exocrine pancreatic function:

A simple method for determination of pancreatic deficiency is very much needed to facilitate genetic and other studies in CF. The evaluation of the synthetic peptide N-benzoyl-L-tyrosyl-p-amino-benzoic acid which is a specific substrate for the proteolytic enzyme, chymotrypsin, and can be administered orally has been continued. If pancreatic enzymes are present, digestion of this tripeptide occurs and PABA is absorbed, metabolized in the liver and excreted in the urine. Quantitation of aromatic amines in urine collected following the administration of a test dose is then performed. A large number of CF patients with and without pancreatic deficiency as well as other patients with pancreatic deficiency due to other unexplained causes of malabsorption as well as normal volunteers have been studied. Results so far have been in agreement with the other tests used to assess digestion and malabsorption including duodenal drainage. These results are encouraging and suggest that this test will be a useful assessment of exocrine pancreatic function. Improved methods for determination of urinary aromatic amines are being investigated in order to make this test a simple office procedure available to all physicians. (Dr. Van S. Hubbard, Dr. Lucille Lester, Dr. Alan Egge, and Dr. Paul di Sant'Agnese, NIAMDD).

4. Endocrine pancreatic function in CF:

It was postulated by previous studies in our branch in conjunction with the CE, NIAMDD (N. Engl. J. Med. 281:451, 1969) that the glucose intolerance in many of the CF patients was due to the increasing fibrosis of the pancreas leading to "strangulation" of the islets and α and β cell dysfunction. To attempt further definition of this hypothesis, four hour oral glucose tolerance tests were performed on 23 CF patients and 8 normal volunteers and glucose and insulin determinations were made. Additionally, 12 of the CF patients including 5 patients with normal exocrine pancreatic function underwent infusions with L-arginine as a stimulation test for glucagon. Only patients with evidence of exocrine pancreatic dysfunction demonstrated depressed levels of insulin and glucagon following these

stimulation tests. Additionally, follow-up of 6 surviving patients from the 1969 study have demonstrated no progression of glucose intolerance or hypoinsulinemia; so, increasing age does not seem to lead to increased endocrine pancreatic dysfunction as previously postulated.

Measurement of glycosylated hemoglobin, (i.e., hemoglobin A1C), thought to be a useful tool in determining which patients need greater help in regulating carbohydrate metabolism, was determined on a pilot study basis in a few of our cystic fibrosis patients without demonstrable glucosuria, but with an abnormal oral glucose tolerance curve. The hemoglobin A1C levels were unusually elevated raising the question of the reliability of this test in the CF population. Further studies will be required before more definite conclusions can be drawn (Dr. Van S. Hubbard, Dr. Paul A. di Sant'Agnese, NIAMDD).

5. Abdominal ultrasonography:

Ultrasound imaging is commonly used to diagnose abnormalities within abdominal organs. On the other hand, non-focal or diffuse parenchymal disease is difficult to diagnose because of the lack of visible contrast between normal and abnormal regions in the organ. Through the development of a digital ultrasound imaging system we have been able to obtain ultrasonic B-scan images of the pancreatic parenchyma in normal and cystic fibrosis patients which can then be numerically analyzed. A prospective clinical trial was performed to determine whether this system could differentiate quantitatively between normal pancreases and the diffusely diseased pancreas of such patients. Both the maximal and minimal echos from the tissue were computed using the digital ultrasound system. Complete segregation of the two groups was achieved by averaging the echo amplitude over a selected region in the image. This work indicates that echo amplitude measurements, selectively retrieved from the B-scan image, can be applied clinically to characterize diffuse pancreatic disease (Dr. Lucille Lester, Dr. Van S. Hubbard, NIAMDD; Dr. Thomas A. Shawker, DR,CC).

6. Echocardiographic Assessment of Cardiac Performance and development of a new echocardiographic scoring system in Cystic Fibrosis (CF).

To characterize the sequence of cardiac changes that accompany the progressive pulmonary disease of CF, we performed M-mode echocardiography on 69 patients. Thirty eight males and 31 females who ranged in age from 6 months to 35 years (mean age 13.1 + 8.4 yrs) followed at NIH (52%) or Children's Hospital of the D.C. (48%) were studied over a 10 month period (March, 1979 to January, 1980). Echocardiographic measurements of right and left heart dimensions and left and right ventricular systolic time intervals were compared to data previously obtained from normals. At the time of the echocardiograms all children had a complete evaluation of their pulmonary status which included a chest roentgenogram and EKG; pulmonary function testing (if age 6 or more), and clinical scoring. Sequential echo studies have been performed to date on 19 patients over a 2 to 15 month follow-up period.

The right ventricular wall thickness was increased in 48 of 69 patients (70%), and the right ventricular dimension was increased in 29 of 69 (42%) of patients, 90% of whom also had increased wall thickness. Increasing right ventricular dilatation was variably associated with alterations of left heart size and function. The RV pre-ejection period/ ejection time ratio measured from the pulmonic valve echo was increased in 9/34 patients (%) suggesting pulmonary artery pressure, and the LV pre-ejection period to ejection time ratio measured from the aortic valve echo was increased suggesting LV dysfunction in 18/60 (%) patients. When echo abnormalities were examined in relation to Shwachman Clinical Score we found that the right ventricular wall thickness was increased in 35% of patients with mild disease (Clinical score (CS) = 81-100), 72% of moderate (CS = 61-80), and in 100% of patients characterized as having moderately-severe (CS = 45-60) or severe (CS = $\leq$45) disease involvement. The right ventricular dimension was increased in 15% of mild; 28% of moderate, 83% of moderately-severe and 100% of patients with severe disease. RV systolic time intervals were abnormal in 50% of patients with moderately severe and 100% of patients with severe disease, and LV systolic time intervals were abnormal in the more severely affected patients in particular those with overt right heart failure.

Based on an apparent sequence of echo abnormalities in the progression from mild to severe disease an echo scoring system was developed. This scoring system which provides an objective, quantitative method for characterizing the cardiac changes that occur in this progressive pulmonary disease correlated very well with Shwachman (r=0.87) and Taussig NIH (r=0.89) clinical scores, chest x-ray scores (r=0.86) and pulmonary function test results in these patients.

Limited sequential data on 19 patients followed for 1-15 months show that stable patients with initial clinical score of 75-90 had little change in cardiac status (echo score); while patients with moderate disease and scores of 55-65 who deteriorated clinically had increasing abnormal (lower) echo scores. Four of 7 patients with scores of 10 or less (normal echo score = 25) died within 6-12 months of the initial evaluation. Echocardiographic studies of patients started on digitalis because of overt failure showed evidence of significantly improved cardiac status ($\uparrow$echo score) in 2 of 4 patients. Further longitudinal studies on this patient group and serial studies to monitor various therapeutic interventions are planned (Dr. L. Lester, Dr. Van S. Hubbard, Dr. Alan Egge, NIAMDD; Dr. Robert Fink, Children's Hosp. of the D.C.).

7. Investigational antipseudomonas antibiotics:

Because of the emergence of drug-resistant strains of Pseudomonas aeruginosa and at times Pseudomonas maltophilia or cepacia in CF patients who have received repeated courses of anti-pseudomonas antibiotics, we undertook a preliminary evaluation of Azlocillin (Az), an investigational semi-synthetic pennicillin, which has excellent reported anti-pseudomonas activity in vitro as well as in vivo in European studies and early clinical trials in the U.S. Ten patients received 18 separate courses of Azlocillin

(300mg/kg/day divided q 4-6 hrly IV doses) in a non-randomized, non-double blind, clinical trial over a 24 month period. The first 3 patients receiving the drug had end-stage pulmonary involvement and Pseudomonas strains highly resistant to the more usual antibiotics (Carbenicillin and Ticarcillin).

We concluded that (1) Judged on the basis of clinical responsiveness Azlocillin appears to be a safe efficacious anti-pseudomonas drug probably similar in activity to Carbenicillin and Ticarcillin especially when used together with an aminoglycoside to treat sensitive organisms. (2) A major advantage of Azlocillin is its use against organisms resistant to Ticarcillin and Carbenicillin and routine sensitivity testing to this and other newly available drugs appears warranted in CF patients. (3) Since some strains of Pseudomonas aeruginosa were observed to be resistant to Azlocillin before ever being exposed to the drug, sensitivity testing should be performed on the patients organisms before administering Azlocillin or other newly available drugs. (4) The low sodium content of Azlocillin (2.1 mEq/gram) as compared to 4.7 mEq/gram of Carbenicillin and 5.2 mEq/gram of Ticarcillin may prove advantageous in patients requiring salt restriction when the suggested doses of 300 mg/kg/day of Azlocillin or Ticarcillin and 600 mg/kg/day of Carbenicillin are administered (Dr. Lucille Lester, Dr. Alan Egge, Dr. Van S. Hubbard, and Dr. Paul A. di Sant'Agnese, NIAMDD).

8. Fatty acid composition of blood and tissue lipids in cystic fibrosis:

We have previously demonstrated that the abnormal fatty acid composition of plasma lipids observed in patients with CF is a secondary effect of the associated pancreatic insufficiency (Hubbard et al, Lancet 2:1302-1304, 1977). This observation has subsequently been confirmed by two other groups (Galabert et al, Lancet 2:903, 1978, Mishler et al, CF Club Abstracts, 1979).We have recently documented (Clinica Chemica Acta 102:115-118, 1980) that the abnormality of the fatty acid composition of erythrocyte phospholipids in CF patients is also only observed in the presence of pancreatic insufficiency. However, several other groups are are still conducting studies relating the defect in fatty acid metabolism to the etiology of CF.

In order to obtain a larger group of patients as well as additional observations, we have continued to investigate the fatty acid composition of plasma lipids in CF patients and obligate heterozygotes. Results of this larger study have shown: 1. That the altered fatty acid composition of plasma lipids does not correlate with the degree of severity of disease, but does correlate with the compliance of patients in taking pancreatic enzyme supplements as well as the degree of malabsorption especially if intestinal resection has been performed. 2. Interfamily comparisons were made to demonstrate that the influence of the diet was not an explanation of the differences. 3. Postpubertal CF patients with pancreatic insufficiency were found to have more severe alterations than pre-pubertal CF patients. 4. There were no differences attributable to the sex of the subject.

In addition, evaluation of other patients with malabsorption syndromes (i.e., Sprue, S/P Billroth II procedures, S/P pancreatectomy) has shown that these patients have alterations of their fatty acid composition of

plasma lipids similar to those observed with the CF patients with pancreatic insuffiency. (Dr. Van S. Hubbard, NIAMDD; Dr. G. Dewey Dunn, Dept. Med., Vanderbilt University, Nashville, Tenn.).

9. Clinical nutrition studies Effects of hypochloremic feedings:

We have initiated a follow-up study of infants who were fed one of two soybean-based formulas deficient in chloride, an essential nutrient. Chloride deficiency can cause metabolic alkalosis, which has been associated with a failure to thrive or a delay in the growth and development of the affected infants.

In July of 1979, a pediatric nephrologist reported to the Food and Drug Administration and the Center for Disease Control three cases of metabolic alkalosis in infants who were being fed only Neo-Mull-Soy, one of the soy formulas. Further investigation has found 118 cases of infants who have had at least one episode of metabolic alkalosis while being fed Neo-Mull-Soy or CHO-Free, the other formula. Analysis of formula preparations found that the products contained one-third the chloride stated on the product label and supplied approximately one-fifth the chloride recommended for infants by the American Academy of Pediatrics.

The study will assess the possible long-term complications of the chloride deficiency and metabolic abnormalities associated with the infant formulas. The studies have been selected in order to rule out other common causes of metabolic alkalosis, especially Bartter's Syndrome, which has symptoms similar to those observed in these infants.

Since only about 1 percent of infants receiving the hypochloremic formulas developed documented problems, the study will try to determine if certain infants have a genetic predisposition to developing metabolic abnormalities during low chloride intake (Dr. Van S. Hubbard, NIAMD & Dr. Artemis P. Simopoulos, OD, DIR; Dr. James W. Hansen, NICHD; Dr. Charles Chang, NINCDS, Dr. Howard Moss, NIMH).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 49000-07 PMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biological and Physiological Studies of Polyamines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: David W. Lundgren, Ph.D. Senior Staff Fellow PMB NIAMDD

COOPERATING UNITS (if any)
Henry M. Fales, Ph.D. LC NHLBI

LAB/BRANCH
Pediatric Metabolism Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH Bethesda, Maryland 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 1	OTHER: 1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The diamine putrescine and the polyamines spermidine and spermine are important metabolites in the early phases of growth and differentiation. An alternate metabolic pathway from putrescine is described for the first time in any biological system. Putrescine is metabolized to 2-pyrrolidone which, in turn, is oxidized to 5-hydroxy-2-pyrrolidone by rat liver.

PHS-6040

Project Description:

Objectives:

The diamine putrescine and the polyamines spermidine and spermine are important compounds in the early stages of growth. Abberrant circulating, urinary and/or tissue levels of these ubiquitous aliphatic amines have been reported in a number of diseases including: cystic fibrosis, many types of cancer, psoriasis, systemic lupus erythematosus, and hereditary muscular dystrophies. A greater understanding of the biochemistry and physiology of these metabolites will increase our knowledge of various processes involved in growth and may enhance our understanding of the underlying basis of one or more of the above mentioned diseases.

Methods:

2-Pyrrolidone Pathway:

The enzymatic steps in spermidine and spermine formation from putrescine have been studied in detail and numerous studies have been carried out attempting to elucidate the manner in which their synthesis is regulated. Considerably less, however, is understood with respect to the metabolic steps, distribution, function and regulation of alternate metabolic pathways from putrescine.

Initial studies revealed that the major product of putrescine metabolism found in spent medium from sliced rat liver is 2-pyrrolidone, a lactam shown for the first time to be synthesized in any biological system (Lundgren, D.W. and Hankins, J. (1978) J. Biol. Chem. 253, 7130-7133). Concurrent with these studies was the report that 2-pyrrolidone is a normal constituent of rat brain. Earlier it was shown that Pseudomonas aeruginosa can utilize 2-pyrrolidone as its sole carbon and nitrogen source. It was of interest, therefore, to determine if in mammalian systems 2-pyrrolidone is a metabolic end product or an intermediate in an alternate metabolic pathway.

To answer the above question, 2-[^{14}C] pyrrolidone was chemically synthesized from [^{14}C] γ-aminobutyric acid and incubated with sliced rat liver in a defined medium. Chromatography of the spent medium revealed that sliced rat liver readily converts 2-pyrrolidone to at least one product.

The unknown 2-[^{14}C] pyrrolidone metabolite was partially purified by chloroform/methanol extraction, activated charcoal chromatography, and two-dimensional thin layer chromatography on silica gel plates. The 2-pyrrolidone metabolite was derivatized with bis(trimethyl) trifluroacetamide and analyzed by gas-chromatography and mass spectrometry. The mass of the molecular ion (245) and fragment ions suggested that the unknown was 5-hydroxy-2-pyrrolidone. Chemically synthesized 5-hydroxy-2-pyrrolidone had an identical mass spectrum and co-chromatographed on silica gel sheets with

the unknown obtained directly from incubation medium. This is the first demonstration of the biosynthesis of 5-hydroxy-2-pyrrolidone in any biological system.

Several effector compounds, but not necessarily the same ones were shown to inhibit or enhance, or both, the conversion of putrescine to 2-pyrrolidone and 2-pyrrolidone to 5-hydroxy-2-pyrrolidone.

Studies are presently being carried out to partially purify and characterize the enzymes in this new metabolic pathway.

It is of interest to note that unlike putrescine, spermidine and spermine which are strong cations at physiological pH, 2-pyrrolidone and 5-hydroxy-2-pyrrolidone carry no net charge.

Significance to Biomedical Research:

The polyamines have been shown to be abnormal in blood and urine of cystic fibrosis patients as well as in some cancer patients. However, the relationship between increased polyamine concentrations and various diseased states remains obscure. A better understanding of the physiology and biochemistry of polyamines may shed light on the nature of cystic fibrosis and/or cancer.

Publications:

Lundgren, D.W. and Fales, H.M.: Metabolism of putrescine to 5-hydroxy-2-pyrrolidone via 2-pyrrolidone. J. Biol. Chem. 255(10): 4481-4486, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 49001-07 PMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Clinical Studies in Cystic Fibrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Paul A. di Sant'Agnese, M.D. Chief PMB NIAMDD

OTHER: Alan C. Egge, M.D. Surgeon PMB NIAMDD
Van S. Hubbard, M.D., Ph.D. Senior Surgeon PMB NIAMDD
Lucille A. Lester, M.D. Senior Staff Fellow PMB NIAMDD

COOPERATING UNITS (if any)
Ronald Kahn, M.D. NIAMDD
Robert Wolf, D.D.S. NIDR
Thomas Shawker, D.R. CC

LAB/BRANCH
Pediatric Metabolism Branch

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

Cystic fibrosis (CF) is a generalized exocrinopathy in which the basic defect is still unknown. Studies have been pursued to determine the characteristics of the disease which are primary and related to a gene defect rather than secondary manifestations. Nutritional assessment of the patients has shown the majority of subjects consume an inadequate number of kilocalories per day to meet their energy requirement and that this observation holds true independent of their degree of pancreatic involvement. Methods of improving nutrient intake have included nutritional counselling and a clinical trial using cimetidine.

New diagnostic methods of evaluating the degree of pancreatic insufficiency have been investigated. Ultrasonography has been used to evaluate abdominal organs as well as cardiac changes which accompany progressive pulmonary disease of CF. These new aids in diagnosing clinical features of CF are especially useful since they offer non-invasive techniques for following longitudinally such functions as cardiac, pancreatic and possibly liver involvement in the CF patient.

Project Description:

Objectives:

These studies are aimed to clarify the significance, incidence, diagnosis and therapy of various abnormalities and complications observed in cystic fibrosis patients of different age groups through the development of clinical studies including the use of experimental diagnostic and therapeutic agents.

1. A survey of the energy and nutritional intake of patients with CF. Although generally it is thought that the CF patient has a voracious appetite and ingests large quantities of food, it is becoming apparent that most CF patients do not fit this description. A written record of all food and beverages consumed over a period of time was obtained from our clinic population. Out of the 24 responses received to date, only 2 patients were consuming 150% or greater of their suggested recommended dietary allowance. Of the remaining 22 patients, 10 (Group A) had intakes of less than 100% of the recommended dietary allowance and 12 (Group B) had intakes between 101 and 150% of the recommended dietary allowance. All of these patients had values for serum vitamin A, vitamin E, calcium, magnesium and (when measured) zinc in the normal range. In our study population, total energy intake did not correlate with either height or weight. Breakdown of the total energy intake into percent contributed by protein, carbohydrate and fat was similar in all groups and in the same proportion as recommended by the United States Dietary Guidelines. Means and ranges for Groups A and B have been tabulated as follows.

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2. The use of cimetidine to improve the malabsorption in patients with CF and pancreatic insufficiency. As an adjunct to pancreatic enzyme supplements in CF patients with exocrine pancreatic insufficiency a 6-week period of treatment of cimetidine was tried. The rationale was that cimetidine, and H_2-receptor antagonist, would decrease gastric acid output and thereby limit gastric inactivation of the supplemental enzymes. In 8 patients studied so far steatorrhea and azotorrhea improved and all had a significant weight gain. No changes were noted in other clinical laboratory parameters and specifically in the plasma lipid fatty acid composition.

However, in a non-CF patient, with pancreatic insufficiency as a result of total pancreatectomy, whose fatty acid composition of plasma lipids originally was almost identical to that observed in CF patients was completely normalized after prolonged cimetidine treatment. Based on this observation as well as the favorable results observed in CF patients on a short term use of this drug, a one year clinical trial utilizing cimetidine as an adjunct to pancreatic enzyme supplement has been initiated. It is interesting in this respect that, in addition to the objective changes mentioned, the patients themselves felt so much improved that they have requested continuation of cimetidine for a longer period of time.

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right ventricular wall thickness was increased in 35% of patients with mild disease (Clinical score (CS) = 81-100), 72% of moderate (CS = 61-80), and in 100% of patients characterized as having moderately-severe (CS = 45-60) or severe (CS = <45) disease involvement. The right ventricular dimension was increased in 15% of mild; 28% of moderate, 83% of moderately-severe and 100% of patients with severe disease. RV systolic time intervals were abnormal in 50% of patients with moderately severe and 100% of patients with severe disease, and LV systolic time intervals were abnormal in the more severely affected patients in particular those with overt right heart failure.

Based on an apparent sequence of echo abnormalities in the progression from mild to severe disease an echo scoring system was developed. This scoring system which provides an objective, quantitative method for characterizing the cardiac changes that occur in this progressive pulmonary disease correlated very well with Shwachman (r=0.87) and Taussig NIH (r=0.89) clinical scores, chest x-ray scores (r=0.86) and pulmonary function test results in these patients.

Limited sequential data on 19 patients followed for 1-15 months show that stable patients with initial clinical score of 75-90 had little change in cardiac status (echo score); while patients with moderate disease and scores of 55-65 who deteriorated clinically had increasing abnormal (lower) echo scores. Four of 7 patients with scores of 10 or less (normal echo score = 25) died within 6-12 months of the initial evaluation. Echocardiographic studies of patients started on digitalis because of overt failure showed evidence of significantly improved cardiac status (↑echo score) in 2 of 4 patients. Further longitudinal studies on this patient group and serial studies to monitor various therapeutic interventions are planned.

7. Investigational antipseudomonas antibiotics. Because of the emergence of drug-resistant strains of Pseudomonas aeruginosa and at times Pseudomonas maltophilia or cepacia in CF patients who have received repeated courses of anti-pseudomonas antibiotics, we undertook a preliminary evaluation of Azlocillin (Az), an investigational semi-synthetic pennicillin, which has excellent reported anti-pseudomonas activity in vitro as well as in vivo in European studies and early clinical trials in the U.S. Ten patients received 18 separate courses of Azlocillin (300mg/kg/day divided q 4-6 hrly IV doses) in a non-randomized, non-double blind, clinical trial over a 24 month period. The first 3 patients receiving the drug had end-stage pulmonary involvement and Pseudomonas strains highly resistant to the more usual antibiotics (Carbenicillin and Ticarcillin).

We concluded that (1) Judged on the basis of clinical responsiveness Azlocillin appears to be a safe efficacious anti-pseudomonas drug probably similar in activity to Carbenicillin and Ticarcillin especially when used together with an aminoglycoside to treat sensitive organisms. (2) A major advantage of Azlocillin is its use against organisms resistant to Ticarcillin

and Carbenicillin and routine sensitivity testing to this and other newly available drugs appears warranted in CF patients. (3) Since some strains of Pseudomonas aeruginosa were observed to be resistant to Azlocillin before ever being exposed to the drug, sensitivity testing should be performed on the patients organisms before administering Azlocillin or other newly available drugs. (4) The low sodium content of Azlocillin (2.1 mEq/gram) as compared to 4.7 mEq/gram of Carbenicillin and 5.2 mEq/gram of Ticarcillin may prove advantageous in patients requiring salt restriction when the suggested doses of 300 mg/kg/day of Azlocillin or Ticarcillin and 600 mg/kg/day of Carbenicillin are administered.

8. Autonomic nervous system dysfunction in patients with cystic fibrosis. Patients with cystic fibrosis exhitib alpha-adrenergic and cholinergic hyperresponsiveness and beta-adrenergic resistance. Alpha and cholinergic responses were tested using pupillary responses to increasing concentrations of phenylephrine and carbamylcholine respectively. The concentration of phenylephrine required to cause pupillary dilation $\leq$0.5 mm was 2.67 $\pm$ 0.07% for 38 healthy control subjects, 1.78 $\pm$ 0.19% for 9 parents of CF patients ($P<0.0001$) and 1.36 $\pm$ 0.12% for 11 CF patients ($p<0.0001$). The concentration of carbamylcholine required to cause pupillary constriction $\geq$ mm was 0.82 $\pm$ 0.04% for 36 healthy control subjects, 0.58 $\pm$ 0.06% ($p<0.01$) for 9 parents, and 0.46 $\pm$ 0.06% ($p<0.001$) for 11 CF patients. Two of 7 CF parents also had a positive response to inhaled methylcholine manifested by a 20% drop in FEV_1 to a dosage of methylcholine below 25 mgm/ml. The rate of infusion of isoproterenol required to produce an increase in pulse pressure $\geq$22 mmHg was 8.04 $\pm$ 0.48 ng/kg/min for 25 normal subjects and 12.38 $\pm$ 1.74 ng/kg/min for 8 CF patients. A similar constellation of autonomic abnormalities has been reported in asthmatic patients. These abnormalities alone are not sufficient to produce either asthma or CF although they may serve to aggravate either condition. Whether there is a genetic basis underlying these autonomic defect in relation to either CF or asthma remains to be proven.

Significance to Biomedical Research:

These studies will aid in the investigations for the basic hereditary defect in CF, the most frequent semi-lethal genetic disease in this country by defining and improving the diagnostic and therapeutic approach to the many manifestations and/or complications of patients with CF of various ages. The approach to counseling about their disease and risk of complications may vary dependent on their age, degree of pancreatic involvement, and development of secondary complications. Additionally, careful studies comparing CF patients with and without clinical exocrine pancreatic insufficiency will allow us to understand more about the role of nutrition in cystic fibrosis and interpret our results more intelligently and thereby possible come closer to the identification of the basic defect.

Publications:

di Sant'Agnese, P.A. and Davis, P.B.: Recent research in cystic fibrosis. Monogr. in Ped. 10: 66-76, 1979.

di Sant'Agnese, P.A. and Davis, P.B.: Ricerca applicata e di base nella fibrosi cistica: proiezione nel futuro. III-Simposio Italiano Sulla Fibrosi Cistica Aggiornamenti Dieterba, Pg. 436-444, 1979.

Hubbard, V.S., Davis, P.B. and di Sant'Agnese, P.A.: Possible hazard of timed-release aspirin in a patient with pancreatic insufficiency. Arch. Int. Med. 139: 1054-1055, 1979.

Davis, P.B. and di Sant'Agnese, P.A.: A review. Cystic fibrosis at forty - Quo Vadis? Ped. Res. 14: 83-87, 1980.

Hubbard, V.S., Davis, P.B., Schwartz, R.H., di Sant'Agnese, P.A. and Gorden, P.: Isolated growth hormone deficiency and cystic fibrosis: A report of two cases. Am. J. Dis. Child. 134: 317-319, 1980.

Hubbard, V.S., Barbero, G. and Chase, H.P.: Selenium and cystic fibrosis. J. Pediatr. 96: 421-422, 1980.

di Sant'Agnese, P.A.: Cystic fibrosis. In Nelson's Textbook of Pediatrics; Vaughan, V.C., McKay, R.J., and Behrman, R.E. (Eds.) W.B. Saunders Co., Publ, Philadelphia, 1979, pp. 1988-2001.

Shawker, T.H., Parks, S.I., Linzer, M., Jones, B., Lester, L.A. and Hubbard, V.S.: Amplitude analysis of the B-scan image: A clinical evaluation of the pancreas. Ultrasonic Imaging 2: 55-66, 1980.

Hubbard, V.S.: Nutrient requirements of patients with cystic fibrosis. Proceendings of the 8th International Congress on Cystic Fibrosis. Ed. Jennifer Sturgess, Pub., Imperial Press Ltd., Mississauga, Ontario, Canada, pp. 149-160, May, 1980.

Davis, P.B., Shelhamer, J.R. and Kaliner, M.: Abnormal adrenergic and cholinergic sensitivity in cystic fibrosis. New Eng. J. Med. 302: 1453-1456, 1980.

Davis, P.B., Hill, S.C. and Ulane, M.M.: Hormone-stimulated cyclic AMP production by skin fibroblasts cultured from healthy persons and patients with cystic fibrosis. Ped. Res. 14: 863-868, 1980.

Hubbard, V.S., Dunn, G.D. and Lester, L.A.: Effectiveness of cimetidine as an adjunct to supplemental pancreatic enzymes in patients with cystic fibrosis. Am. J. Clin. Nutr. (in press).

Davis, P.B., Hubbard, V.S. and di Sant'Agnese, P.A.: Low sweat electrolytes in cystic fibrosis. Am. J. Med. (in press).

Wolf, R.O. and Hubbard, V.S.: Serum salivary and pancreatic amylase. Pediatr. Res. (in press).

Lester, L.A., Egge, A.C., Hubbard, V.S., Camerini-Otero, C.S. and Fink, R.J.: Echocardiography in cystic fibrosis: A proposed scoring system. J. Pediatr. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-49003-04 PMB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotides and the Autonomic Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Pamela B. Davis, M.D., Ph.D. Clinical Associate PMB NIAMDD

COOPERATING UNITS (if any)

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Pediatric Metabolism Branch

SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 49004-01 PMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Fatty Acids and Membrane Function in Cystic Fibrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Van S. Hubbard, M.D., Ph.D. Senior Surgeon PMB NIAMDD

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2	1	1

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The fatty acid composition of blood and tissue lipids of patients with cystic fibrosis has been shown to be abnormal. The predominant alterations include a decreased linoleic acid and increased palmitoleic and oleic acids. These alterations are primarily due to the malabsorption and maldigestion associated with pancreatic insufficiency in the cystic fibrosis patient and are not corrected by exogenous pancreatic enzyme supplementation. The major unsaturated fatty acid within phospholipids, and thus within membrane lipids is linoleic acid. Decreased linoleic acid content as observed in most cystic fibrosis patients is thus associated with decreased unsaturation in tissue phospholipids and membranes. This characteristic may be related to alterations in membrane function as well as altering prostaglandin synthesis. Current work is aimed at delineating the role of altered fatty acid composition of blood and membrane lipids as a cause of decreased responsiveness of leukocytes to stimulation by isoproterenol, alterations in the recognition or interaction of the beta-receptors in CF tissues, and alteration in the availability of arachidonic acid for the biosynthesis of prostaglandin.

Project Description:

Objectives:

The fatty acid composition of plasma and tissue lipids of patients with cystic fibrosis has been shown to be abnormal. Our work during the past couple of years had documented that this generalized observation is a result of the malabsorption and maldigestion associated with pancreatic insufficiency in the cystic fibrosis patient and cannot be corrected by any current routine therapeutic intervention. Since 85-90% of the cystic fibrosis patients are observed to have this abnormality of fatty acid composition, it is important to determine how this altered fatty acid composition influences both *in vitro* and *in vivo* studies from patients with cystic fibrosis.

Fatty acid composition of blood and tissue lipids in cystic fibrosis:

We have previously demonstrated that the abnormal fatty acid composition of plasma lipids observed in patients with CF is a secondary effect of the associated pancreatic insufficiency (Hubbard et al, Lancet 2:1302-1304, 1977). This observation has subsequently been confirmed by two other groups (Galabert et al, Lancet 2:903, 1978, Mishler et al, CF Club Abstracts, 1979).We have recently documented (Clinica Chemica Acta 102:115-118, 1980) that the abnormality of the fatty acid composition of erythrocyte phospholipids in CF patients is also only observed in the presence of pancreatic insufficiency. However, several other groups are are still conducting studies relating the defect in fatty acid metabolism to the etiology of CF.

In order to obtain a larger group of patients as well as additional observations, we have continued to investigate the fatty acid composition of plasma lipids in CF patients and obligate heterozygotes. Results of this larger study have shown: 1. That the altered fatty acid composition of plasma lipids does not correlate with the degree of severity of disease, but does correlate with the compliance of patients in taking pancreatic enzyme supplements as well as the degree of malabsorption especially if intestinal resection has been performed. 2. Interfamily comparisons were made to demonstrate that the influence of the diet was not an explanation of the differences. 3. Postpubertal CF patients with pancreatic insufficiency were found to have more severe alterations than pre-pubertal CF patients. 4. There were no differences attributable to the sex of the subject.

In addition, evaluation of other patients with malabsorption syndromes (i.e., Sprue, S/P Billroth II procedures, S/P pancreatectomy) has shown that these patients have alterations of their fatty acid composition of plasma lipids similar to those observed with the CF patients with pancreatic insuffiency.

Fatty acid composition in membrane function:

To check the possibility of altered fatty acid composition contributing

to the observations of decreased responsiveness of leukocytes to stimulation by isoproterenol as measured by the formation of cyclic AMP in cells derived from cystic fibrosis patients, analyses of the fatty acid composition of the phospholipid component of mononuclear cells were performed. Mononuclear cells were obtained from four cystic fibrosis patients with pancreatic insufficiency and one cystic fibrosis patient without pancreatic insufficiency. Fatty acid composition of the phospholipid component of the lipid extract of these crude monocyte preparations resulted in an increased percentage of arachidonic acid in all samples. The fact that this alteration of the percentage of arachidonic acid was found in both groups of CF patients suggests that it cannot be totally attributed to malabsorption and thus makes the observation more intriguing.

Hirata et al (PNAS 75:1718, 1978, PNAS 76:368, 1979) have shown that in the erythrocyte, reticulocyte and other cells, there is a transmethylation pathway present in plasma membrane which is catalyzed by two separate enzymes. Their investigations have shown that if there is a defect in this pathway, an uncoupling phenomenon occurs between the stimulation of the beta receptor and the formation of cyclic AMP. Additionally, if there is a defect in the transmethylation pathway, one would predict that the concentration of phosphotidylethanolamine and phosphotylserine on the inner side of the plasma membrane would be increased along with a decrease in the biosynthesis and subsequent concentration of phosphotidylcholine on the outer side of the membrane. These events would account for an increase in the arachidonic acid in phospholipids derived from such cells since arachidonic acid is the predominant fatty acid in the C-2 position of phosphotidylserine and phosphotidylethanolamine and in the normal situation the fatty acid in the C-2 position of phosphotidylcholine on the outer side of the membrane is cleaved by phospholipase A-2 to yield lysolecithin. Preliminary studies in patients both with and without pancreatic insufficiency indicate that there may be a defect in this transmethylation pathway of phospholipids when using an enzyme preparation derived from the leukocytes of these patients. These studies are continuing in collaboration with Dr. Hirata for futher delineation of this possible enzymatic defect.

Significance to Biomedical Research:

The apparent defect in the transmethylation pathway in enzyme preparations derived from cells of CF patients is consistent with and a partial explanation for two other experimental observations in such cells (1) decreased responsiveness to isoproterenol stimulation and (2) increased percentage of arachidonic acid in the phospholipid fraction. Extension of these studies will enable us to determine if these observations are associated with the basic defect responsible for cystic fibrosis.

Publications:

Hubbard, V.S. and Dunn, G.D.: Fatty acid composition of erythrocyte phospholipids from patients with cystic fibrosis. Clin. Chim. Acta 102: 115-118, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 49005-01 PMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Follow-up of Hypochloremic Infant Formula Feedings

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Three cases of Bartter-like syndrome in infants were reported to the Center for Disease Control on July 26, 1979. These infants were less than 10 months of age, failed to gain weight, had poor appetites, and were observed to have hypochloremia, hypokalemia, and varying degrees of alkalosis. Subsequently approximately 118 infants have been documented to have experienced the same constellation of symptoms. The common denominator in all these cases is thought to be the consumption of the same brand of soy-base formula, Neo-Mull-Soy. It was learned that this formula contained deficient amounts of chloride and infants using this formula as the sole or predominant source of food developed a severe chloride deficiency with other associated metabolic alterations. Long-term effects of chloride-deficient feedings in humans is not known and therefore a follow-up of these infants has been initiated.

Project Description:

Objectives:

This study will assess the possible long-term complications of chloride deficient formula feedings and metabolic abnormalities associated with these infant formulas. This study will also attempt to determine if certain infants within this group had a pre-disposition to the development of metabolic abnormalities during a low chloride intake.

Methods:

We have initiated a five-year follow-up study of infants who were fed one of two soy-bean based formulas deficient in chloride, an essential nutrient. Chloride deficiency can cause metabolic alkalosis which has been associated with a failure to thrive or a delay in the growth and development of affected infants. In July, 1979 a pediatric nephrologist reported to the Food and Drug Administration and to the Center for Disease Control 3 cases of metabolic alkalosis in infants who were being fed only Neo-Mull-Soy, one of the soy formulas. Further investigations have found at least 118 cases of infants having episodes of metabolic alkalosis (many having a blood pH of 7.60 or greater) associated with feedings of Neo-Mullsoy or CHO-Free, the other formula. Analysis of formula preparations has shown that the products contained one-third of the chloride stated on the product lable and supplied approximately one fifth of the chloride recommended for infants by the American Academy of Pediatrics.

Studies have been selected in order to rule out other common causes of metabolic alkalosis especially Bartter's syndrome which has symptoms similar to those observed in these infants. These studies will include clinical evaluation with emphasis on growth and development, and detailed neurological and psychological examination. Laboratory evaluation of the metabolic status of the infants will also be monitored.

Only 1% of infants receiving the hypochloremic formulas developed documented problems. Thus, this study will try to determine if these infants had a genetic predisposition to developing metabolic abnormalities during low chloride intake.

Significance to Biomedical Research:

Since the occurrence of the chloride deficient feedings in these infants has been likened to an "experiment in nature", it offers an unique opportunity to study the important role of chloride in growth and development in humans. The time of the chloride deficiency in relationship to the rapid rate of growth which occurs in the child at such a time makes further understanding of all nutrient requirements of utmost importance.

ANNUAL REPORT SUMMARY

CLINICAL HEMATOLOGY BRANCH

I. Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

A. Artifactual Indications of Chemiosmotic Secretion Resulting from the Use of Anion Transport Blocking Agents with Platelets

Following the demonstration that ATP-evoked epinephrine release from isolated adrenal chromaffin granules depended on anion permeation that could be inhibited by anion transport blocking drugs such as probenecid, pyridoxylphosphate, SITS, suranim and others, it was considered that anion transport might be the key to secretion by intact cells. In the case of platelets, the transport blocking agents essentially duplicated results obtained on chromaffin granules. That is, dose-dependent inhibition of serotonin secretion was similar, and increasing extracellular osmotic strength progressively inhibited secretion. Although platelet secretion was not Cl^- dependent, it was markedly sensitive to pH and the lower the pH, the relatively more effective were anion transport blockers, suggesting that OH^- was the transported ion.

Our interest in sequential biochemical events in platelet secretion led us to compare prostaglandin synthesis in uninhibited platelets and platelets inhibited by anion transport blockers. If inhibition occurred at the end of cellular secretory events as suggested by the chemiosmotic theory, then the initial biochemical responses of platelets to secretory stimuli should have remained intact. These initial responses involve activation of phospholipase which produces arachidonic acid (AA) from platelet phospholipid and subsequent peroxidation products of AA to yield stable malondialdehyde (MDA). All of this transpires before secretion takes place. To our surprise, MDA formation was always inhibited in proportion to secretion when anion transport blockers were present.

Experiments designed to measure free and bound agonist when thrombin was used as the stimulus for platelet release in the presence and absence of anion transport inhibitors indicated clearly that these inhibitors interfere with attachment of agonist to the cell. Actually anion transport inhibitors are known to be highly reactive with cell membrane proteins and probably exert their transport inhibition by interference at the site of membrane proteins that act as anion gateways. In the case of platelets the inhibitors may bind to receptors for thrombin which is the one agonist that permits accurate assessment of inhibition. Secretion following other stimuli such as ADP and A23187, which appeared to be inhibited by anion transport blockers in the original experiments, was not inhibited in the recent studies. Work directed at determining the platelet protein receptor that might be involved in reactions with anion transport blockers, strongly suggests that it is platelet-associated fibrinogen, known to be of importance in this cell's secretory reaction.

B. Evaluation of Phospholipid Methylation as a Biochemical Event Initiating Platelet Secretion

There has been considerable interest recently in phospholipid methylation

and its possible role in secretory and chemotactic mechanisms. Recent studies have shown that the mobility and distribution of receptors on the cell surface are modulated by stimuli that evoke cellular secretion for example by mast cells, cellular mitogeneics in the case of lymphocytes, and phagocytosis by polymorphonuclear leukocytes. Phospholipid methylation can be inhibited competitively by S-adenosyl-L-homo cysteine which accumulates intracellularly when deazadenosine and homocysteine are in cellular incubation mixtures. In association with J. Axelrod we have studied the effects of inhibition of phospholipid methylation on platelet function in vitro. In contrast to findings on mast cells, lymphocytes, and polys, platelet function was not altered. Serotonin secretion, malondialdehyde formation, aggregation, and clot retraction remained normal in response to ADP, thrombin, A-23187, collagen and arachidonic acid stimulation. The apparently exceptional behavior of platelets is as yet not fully explained but may be related to the unusual phospholipid content of platelet membranes. Previous studies of methylation have involved primarily phosphatidylethanolamine whereas phosphatidylcholine predominates in platelets. These observations raise the question as to whether an increase in membrane fluidity is invariably a requirement for cellular response.

C. Preparation of Secretory Granules from Platelets

Availability of isolated platelet granules would provide a means of evaluating subcellular mechanisms of platelet secretion. Platelet granules have not been obtainable in pure form primarily because usual methods of cellular disruption cause release of granular material. The approach to isolation of granules that we used involved: 1/ introduction of reversible inhibitory agents and factors during purification steps; namely, low pH, 2-deoxy glucose, apyrase and rotenone 2/ evaluation of ultrasonication, homogenization, glycerol lysis, nitrogen cavitation, freeze-thawing, and the French pressure cell 3/ cell organelle markers such as lactic dehydrogenase for cytoplasmic material, 5-hydroxytryptamine for granular content, ATP for effectiveness inhibition of release, beta galactosidase for lysosomes, monamine oxidase for mitachonria the Pl^{A1} antigen for plasma membrane 4/ electronmicroscopy to identify organelles and 5/ sucrosemetrizamide gradients for isopycnic separation of subcellular structures.

Best results were obtained with Frence pressure cell lysis, yielding 18-32% of intact secretory dense granules which on gradients concentrated between mitochondria and alpha granules, distinctly separated from plasma membranes which were the most buoyant. Of special interest was the finding that a specific plasma marker, Pl^{A1} antigen, was concentrated along with secretory granules. Whether this reflects interaction and fusion of granules with plasma membranes or similar antigenic content of the two types of membranes remains to be answered. An attractive theoretical possibility is that granular membranes may be indistinguishable from plasma membranes and may be incorporated into them after fusion and fission.

D. Kinetic Analysis of Platelet Survival

Many models for platelet survival have been proposed from the shapes of disappearance curves of labeled cells and biological observations on

platelet aging. Analyses to date have included senescent and random-destructive components only. None of the analyses provide for the observation that platelets survive for a shorter time in thrombocytopenic patients than in those with normal platelet levels. It has been assumed that platelet utilization is greater in thrombocytopenics because of some unspecified deficit.

In our experience, recovery of transfused platelets in thrombocytopenic patients is normal; and although survival is shortened, it is proportional to the total number of platelets transfused. We found that when sufficient platelets are transfused to bring the recipient's count to normal, a circumstance not often effected clinically, platelet survival in the recipient becomes normal. Assuming a component of fixed-loss added to senescent loss permits generating survival curves mathematically that fit the observations in thrombocytopenic as well as normal individuals. If n_t = platelet level at time, t, in days, n_o = the initial platelet level, a = rate constant for senescent loss and b = the constant for fixed loss, then

$$n_t = n_o(1-at) - bt$$

and

$$t = \frac{n_o - n_t}{n_o a + b}$$

Thus at large n_o, b (assumed to be on the order of $10{,}000/mm^3$/day, becomes insignificant and t is fixed; and at relatively small values for n_o, the value for t decreases.

E. Thrombosis in Homocysteinuria

Patients with homocysteinuria are highly susceptable to thromboembolic disorders. It is considered that abnormal interaction between endothelium and platelets is responsible for this complication. Some investigators have reported markedly shortened platelet survival in this disease and reversal of shortened survival by treatment with dipyridamole. We have not found shortened platelet survival in 6 patients with homocysteinuria. In the past 2 years we observed 2 patients with this disease who developed thrombophlebitis and pulmonary embolism while taking dipyridamole and aspirin prophylactically; and this year we observed a third on "antiplatelet" therapy. We have recommended in a report in the N. Eng. J. Med. that homocysteinuric patients receive anticoagulant therapy (coumadin or heparin) directed at reducing clotting factors rather than "antiplatelet" therapy.

F. Plateletinhibitory Drugs of Clinical Importance

A number of drugs known to inhibit platelet function may have value in preventing recurrence of thrombosis or coronary insufficiency. We have compared the relative effectiveness of these drugs (sulfinpyrazone, ibuprofen, indometacin, verapamil and aspirin) as *in vitro* inhibitors of platelet function which may be beneficial and as *in vivo* inhibitors of vasodilation which may be harmful. This work was done in association with the Cardiology Branch of NHLBI which evaluated the *in vivo* vaso responses. These studies indicate that effects of the various drugs on platelets and the vasculature

have the same dose response; so far none have shown platelet selectivity.

II. Study of Immunology of Blood Cell Dificiencies

A. Platelet Immunology

During the past year we have studied 25 sera from patients with suspected posttransfusion purpura or from mothers whose infants were thought to have isoimmune neonatal purpura. We have documented the cause of disease in 20 instances. Two new platelet antigen groups were defined with 1 maternal and one post-transfusion antiserum.

One platelet preparation on our panel was of special interest for its unique reaction with anti-$P1^{A1}$ antisera. This individuals platelets had complement-fixing and absorption reactions typical of over 1000 positive donors except for reactions with one of six examples of anti-$P1^{A1}$ with which it did not react. This unique exception suggests that there may be variants in the well documented $P1^{A1}$ platelet antigen that could account for certain immunologic discrepancies that appear with literature. The family of the platelet donor and the differentiating antiserum are under further study.

B. Treatment for Drug Purpura and Isoimmune Purpura

We have shown by extensive in vitro immunologic analyses that isoimmune post-transfusion purpura and drug purpura are most likely caused by adsorption of antigen-antibody complexes rather than direct attachment of antibodies to platelets. In drug purpura, simply stopping the drug leads to cure, but there may be a delay of several days before all drug or drug-antibody complexes decline to innocuous levels in the circulation. Several days after cardiac surgery a patient became acutely thrombocytopenic owing to a quindine antibody. We demonstrated for the first time that plasmapheresis could be curative in drug purpura. At the end of the plasma exchange which took several hours, no further purpura occurred and platelets began returning to the circulation. The unusually prompt remission suggests that continued circulation of antigen-antibody complexes may prolong recovery in cases treated by drug withdrawal alone. An additional case of posttransfusion purpura cured by plasmapheresis further substantiates the effectiveness of this form of therapy and also suggests that circulating antigen-antibody complexes are the cause of the disease.

III. Mechanisms of Hormone and Transmitter Secretion

A. Mechanisms of Calcium Action in Exocytosis

We have recently proposed that synexin, an adrenal medullary protein that causes Ca^{2+}-dependent aggregation of isolated chromaffin granules, might be the intracellular receptor for Ca^{2+} in the process of exocytosis. Antibodies to bovine adrenal synexin have been produced in rabbits, and cytoimmunochemical analysis has shown the synexin antigen to be localized generally to the cytoplasm of the chromaffin cell. The antigen has also been localized to bovine blood leukocytes and platelets by the

same method. Synexin activity has also been detected in soluble extracts of bovine brain, salivary gland and liver, and in human platelets. Synexins from bovine liver and adrenal medulla have been purified in substantial quantity and shown to be identical by a number of quantitative physical and chemical criteria. Sites of synexin association have now been detected on the inner aspects of chromaffin cell plasma membranes, and have been found to be quite analogous to Ca^{2+}-dependent synexin association sites previously described on chromaffin granule membranes. We presently feel that synexin may act to promote Ca^{2+}-dependent secretion by joining secretory vesicle membranes either to plasma membranes or to other secretory vesicle membranes as during compound exocytosis.

B. Chemiosmotic Regulation of Chromaffin Granule Lysis

We have presented evidence that exocytosis from bovine chromaffin and parathroid cells, as well as human platelets, may be related to a chemiosmotic lysis process of secretory granules juxtaposed to plasma membranes. The chemiosmotic lysis of granules can be studied in vitro with isolated chromaffin granules, where lysis occurs as a consequence of an electrogenic inward H^+ current (ATPase) and coinadent inward flow of Cl^- as a counter ion. We have therefore devoted some considerable attention to the role of the proton-pumping ATPase and the necessary permeant anions. In previous work we were able to detect anion transport sites on granule membranes that were sensitive to a variety of anion transport blocking drugs. We have now detected a second type of anion transport site that can be detected by studying the anion dependence of valinomycin-induced granule lysis. We have found that this second site has the same anion selectivity as the drug-sensitive transport site but lacks the drug sensitivity. We have also found that the granule membrane ATPase activity is anion activated, and that this anion activation was also competitively inhibited by the anion transport blocking drugs. These different sites may be critically related to exocytosis from chromaffin cells inasmuch as secretion from chromaffin cells has been found to be sensitive to different anions but insensitive to the blocking drugs.

C. Biosynthesis and Packaging of Adrenaline

Adrenaline is synthesized by the sequential action of dopamin-β-hydroxylase (DBH) upon intragranular dopamine and by the action of phenyl-N-methytransferase (PNMT) upon noradrenaline moved out side the granule. The granule contains a number of factors found necessary for DBH function such as an acid pH (~5.7) and ascorbic acid (13mM), but until now no catalase was found which was otherwise necessary in the in vitro experiment to destroy the H_2O_2 generated by DBH action. We have now discovered a small but significant catalase activity associated with the intact chromaffin granule which can be shown to be associated with a more dense particle ($\rho \simeq 1.18$) when the granule is lysed. It is possible that chromaffin granules have peroxisone-like structures associated with them otherwise obscured by their naturally dense cores. We have also devised a rapid radiometric assay method for measuring PNMT activity in a single scintillation vial. After synthesis of adrenaline, the new adrenaline then moves

back into the granule by the specific transport system in exchange for another endogenous catecholamine, perhaps noradrenaline.

D. Mechanism of Acetylcholine Secretion from Cholinergic Nerve Terminals

Synaptosomes from cephalopod brain (squid or octopus) are over one-hundred-fold enriched in acetylcholine over analogous preparations from mammalian brain, and they seem to represent a system suitable for chemical analysis of processes occurring, for example, at cholinergic neuromuscular junctions. There is presently a controversy over whether acetylcholine release is truly by exocytosis, and we have examined this question by asking whether other vesicle components such as ATP were also secreted when the synaptosomes were stimulated with veratridine. We found that veratridine could cause release of up to 50% of the endogenous ATP stores from squid synaptosomes by a process that was sensitive both to low [Ca^{++}] and to the specific veratridine inhibitor tetrodotoxin (TTX). We concluded that the data supported an exocytotic mechanism for acetylcholine release, but that the anatomic identity of the acetylcholine and ATP compartments remained to be discovered.

E. Phosphorylation of Specific Proteins in Cholinergic Nerve Terminals During Secretion

It is believed that phosphorylation and dephosphorylation of specific proteins may be one way signals are transmitted from one place to another in cells and nerve ternimals, and we have examined this concept in the relatively homogeneous cholinergic nerve terminal preparation from squid brain under control and stimulated conditions. One of the most useful and fascinating properties of the squid system is the ready accessibility of pure axoplasm from the giant axon, thereby allowing ready accessibility to both homogenous axons and nerve terminals in the same system. We found that the intact axon was characterized by a specific high molecular weight neurofilament protein that was the specific acceptor of radioactive phosphate presented either as ^{32}P or [γ-^{32}P]-ATP. By contrast, the major phosphorylated species in purified synaptosomes was a 47,000 MW component, probably located close to or associated with the synaptosomal plasma membrane. Exposure of the synaptosome to Ca^{++}, either by adding a depolarizing condition such as high [K^+] or veratridine, or by adding A23187 (the calcium ionophore) caused the 47K synaptosomal protein to become dephosphorylated. By contrast, the neurofilament protein, under the same conditions, became digested by a specific Ca^{2+}-activated protease. Thus the mechanisms for regulating phosphorylation under similar conditions differed for both axons and terminals. It is possible that the discret differences in major proteins and phospho-proteins maybe related to synaptic transmission or other processes occuring at cholinergic terminals.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 51,000-22 CHB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Study of Immunology of Blood Cell Deficiencies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. Raphael Shulman	Chief	CHB, NIAMDD
Other:	James V. Jordan, Jr.	Clinical Associate	CHB, NIAMDD
	Ellen Goldfarb	Biologist	CHB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.0	.25

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Autoimmunity, drug antibodies, platelet antigens, isoimmunization, transplantation antigens, hepatitis, hemagglutination tests, thrombocytopenia, blood coagulation inhibitors.

Project No. AM 51,000- 22 CHB

Project Description:

Objectives:

To study the nature of immunologic reactions which result in formation of antibodies against autologous blood cells, and to determine the significance of immunity in development of "idiopathic" blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies, and drug haptenes and the physiologic processes which result in sequestration of cells with attached antibodies. In recent years our work on identifying inherited leukocyte and platelet isoantigens has led to a study of the significance of these antigens in hetero- and homo-transplantation. Immunologic techniques have been used to develop a number of clinically practical diagnostic tests for certain infectious diseases and drug addiction.

Methods Employed:

Techniques of quantitative immunochemistry, including preparation and physiocochemical characterization of purified antibodies and antigens, microanalyses for nitrogen, histamine, and alkaloid drugs, quantitative measurements of complement fixation, cellular agglutination and precipitin reactions, immunoelectrophoresis, methods of provoking antibody responses in man and animals, and isotopic and fluorescent labeling applied to antigens and antibodies. Tissue culture techniques, red cell agglutination and techniques and lymphocyte transformation tests, including radioautography. Methods of hemagglutination involving attachment of various antigens to erythrocytes by chemical coupling.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 51,001- 22 CHB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. Raphael Shulman	Chief	CHB, NIAMDD
Other:	James V. Jordan, Jr.	Clinical Associate	CHB, NIAMDD
	Ellen K. McGlade	Masters Candidate, Univ. of MD	CHB, NIAMDD

COOPERATING UNITS (if any)

J. Axelrod	Laboratory Chief	LCS, NIMH
N. Davenport	Senior Investigator	CB, NHLBI
J. Schulman	Senior Investigator	NPMB, NICHD

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hemophillia, coagulation mechanism, clotting factor assays, clotting factor purification, platelet physiology, platelet metabolism, pharmacology of platelet-inhibitory drugs.

Project Description:

Objectives:

Study of the reactions and interactions of coagulation factors *in vitro* and *in vivo* to define further the nature of the blood coagulation mechanism, to determine factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis, and to develop better forms of therapy for these diseases.

Methods Employed:

Methods of protein purification and characterization, techniques of enzymology applied primarily to proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactivity labeled substrates.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM51, 002- 07CHB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanisms of Hormone and Transmitter Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Harvey B. Pollard	Senior Investigator	CHB, NIAMDD
OTHER:	Christopher J. Pazoles	Senior Staff Fellow	CHB, NIAMDD
	Carl E. Creutz	Senior Staff Fellow	CHB, NIAMDD
	Janet H. Scott	Guest Worker	CHB, NIAMDD
	Velia Fowler	Guest Worker	CHB, NIAMDD
	Samuel S. Stopak	Guest Worker	CHB, NIAMDD
	Oren Zinder	Visiting Scientist	CHB, NIAMDD
	Avner Ramu	Visiting Scientist	POB, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.25	5.25	1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Exocytosis of neurotransmitters, hormones and other cell secretory products is believed to occur by calcium-dependent fusion of secretory vesicles with the plasma membrane and by subsequent breakage (fission) of the barrier separating the vesicle interior from the extracellular medium. We have continued our study on synexin, a protein we recently discovered, that can cause the formation of pentalaminar complexes between adjacent chromaffin granule membranes and chromaffin cell plasma membranes in a Ca^{++}-dependent manner. This protein may regulate the fusion step in exocytosis, and we have found the activity in a number of secretory organs. More evidence has been accumulated supporting the concept that the fission step in exocytosis may be energized by local osmotic lysis of the fused secretory granule. Finally, evidence that phosphorylation events maybe of importance in secretion, at least in cholinergic terminals, has been obtained.

Project Description:

Objectives:

The object of our work has been to discover the molecular bases for regulation of cellular secretion of hormones and transmitters by exocytosis. Our approach has been to search for factors mediating calcium action, the formation of complexes between secretory vesicles and plasma membranes and the final breakage (fission) of this complex leading to the ultimate release event of exocytosis.

Methods Employed:

The methods we have employed include cell fractionation, ultracentrifugation, light and electron microscopy, enzymology, scintillation counting, light scattering, fluorescence spectroscopy and nuclear magnetic resonance.

Publications:

Pollard, H.B., Pazoles, C.J., Creutz, C. E. and Zinder, O.: Thechromaffin granule and possible mechanisms of exocytosis. Int. Rev. Cytol. 58: 159-197, 1979.

Zinder, O. and Pollard, H.B.: The chromaffin granule: A functional model for exocytosis. In Lovenberg, W. and Youdin, M. (Eds.): Essays in Neurochemistry and Neuropharmacology. Oxford,Permagon Press, 1980,pp. 125-162.

Pollard, H.B., Stopak, S.S., Pazoles, C. J. and Creutz, C. E.: A simplified, one-step radiometric assay for phenyl-N-methyl transferase in adrenal medullary cell extracts. Anal. Biochem. 99: 281-282, 1979.

Pollard, H.B., Pazoles, C. J. and Creutz, E. E.: Evidence in support of a chemiosmotic mechanism for exocytosis from platelets, parathyroid and chromaffin cells. In Kopin, I. and Usdin, E. (Eds.): Catecholamines: Basic and Clinical Frontiers. Oxford, Permagon Press, 1979, pp. 328-330.

Zinder, O. and Pollard, H.B.: Regulation of adenylate cyclase in chromaffin granule membranes. In Kopin, I. and Usdin, E. (Eds.): Catecholamines: Basic and Clinical Frontiers. Oxford, Permagon Press, 1979, pp. 343-346.

Pazoles, C. J., Creutz, C. E. and Pollard, H. B.: Identification of anion transport sites in chromaffin granules and their involvement in an osmotic mechanism for epinephrine release. In Kopin, I. and Usdin, E. (Eds.): Catecholamine: Basic and Clinical Frontiers. Oxford, Permagon Press, 1979, pp. 319-321.

Creutz, C.E., Pazoles, C. J. and Pollard, H.B.: Synexin: An adrenal medullary protein that may be an intracellular receptor for Ca^{++} in the process of exocytosis. In Kopin, I. and Usdin, E. (Eds.): Catecholamines: Basic and Clinical Frontiers. Oxford, Permagon Press, 1979, pp. 346-349.

Pollard, H. B. and Pappas, G. D.: Veratridine-activated release of adenosine-5'-triphosphate from synaptosomes: Evidence for calcium dependence and blockade by tetrodotoxin. Biochem. Biophys. Res. Comm. 88: 1315-1321, 1979.

Pazoles, C. J., Claggett, C. E., Creutz, C. E., Pollard, H. B. and Weinbach, E. C.: Identification and subcellular localization of catalase activity in bovine adrenal medulla and cortex. Arch. Biochem. Biophys. 200: 434-443, 1980.

Pant, H., Pollard, H. B., Pappas, G. D. and Gainer, H.: Phosphorylation of specific, distinct proteins in synaptosomes and axons from squid nervous system. Proc. Natl. Acad. Sci. USA 76: 6071-6075, 1979.

ANNUAL REPORT SUMMARY OF THE GENETICS AND BIOCHEMISTRY BRANCH,

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

In the area of molecular genetics and gene expression, Drs. Camerini-Otero and Zasloff have studied the nucleoprotein structure of the Herpes thymidine kinase gene introduced into mouse fibroblasts (Ltk^-, $aprt^-$) by means of DNA-mediated gene transfer. They have shown that this gene, when expressed in the recipient cells and present in a single copy, is packaged into nucleosomes.

Dr. Camerini-Otero is investigating the mechanism by which genes so introduced become integrated and expressed in a variety of cells. The technique of biochemical transformation is also being tried as an assay for the purification of genes of interest such as those coding for hypoxanthine phosphoribosyltransferase and histocompatibility antigens.

Dr. Zasloff has continued the study of the organization of $tRNA_i^{met}$ genes in the human genome. He has isolated and characterized four recombinant phages bearing fragments of DNA which include the $tRNA_i^{met}$ gene. The sequence and organization of the DNA adjoining this gene are under investigation.

In the area of biochmical genetics Dr. Neufeld is extending the study of lysosomal enzymes in human cells to the proteolytic processing of the higher molecular weight precursor forms of these enzymes. Such processing probably occurs within lysosomes, by the action of a thiol protease; however, it can be mimicked by the action of trypsin or chymotrypsin. The structure and function of the extension peptide(s) are under investigation.

Dr. Robbins is studying the glycosylation and translocation of newly synthesized lysosomal enzymes using mutant CHO cells. She found that synthesis of the phosphorylated recognition marker on the enzyme requires prior transfer of glucose to the oligosaccharide-lipid precursor of the carbohydrate chains. By developing mutants defective in mannose 6-phosphate receptor, she demonstrated that the surface receptor required for the uptake of exogenous enzyme also mediates the translocation of newly synthesized enzymes to lysosomes.

In the area of clinical genetics, Dr. Zasloff is studying mechanisms by which mucoid strains of *Pseudomonas aeruginosa* colonize the respiratory tract of patients with cystic fibrosis. The mucoid phenotype confers a selective resistance to lysis induced by several agents, including many l tic and lysogenic bacteriophages as well as several aeruginocins. Isolation of bacteriophage from the bronchopulmonary tree of patients with cystic fibrosis and further characterization of phage-bacterial interactions are in progress.

Dr. Zasloff has also undertaken a study of fibrodysplasia ossificans progressiva, an autosomal dominant disorder in which heterotopic bone forms

within connective tissue that surrounds skeletal muscle. Studies are focused on the basic cellular defect, the natural history of the disease and therapeutic intervention.

In collaboration with clinical investigators at Johns Hopkins University, Dr. Neufeld is studying the potential of plasma exchange as a means of replacing the deficient enzyme (iduronate sulfatase) in the Hunter syndrome.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52000-13 GBB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Biochemical Lesions in the Genetic Mucopolysaccharidoses and Mucolipidoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elizabeth Neufeld	Biochemist & Branch Chief	GBB NIAMDD
OTHER:	Amos Frisch	Visiting Fellow	GBB NIAMDD
	Clara Hall	Research Chemist	GBB NIAMDD
	Rachel Myerowitz	Guest Worker	GBB NIAMDD
	Christina Puchalski	Chemist	GBB NIAMDD
	Pamela Robey	Guest Worker	GBB NIAMDD
	James Scott	Biologist	GBB NIAMDD
	Bernard Weissman	Guest Worker	GBB NIAMDD

COOPERATING UNITS (if any)
John Kennedy Institute, Baltimore, Maryland (Dr. Hugo Moser)

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION National Institute of Arthritis, Metabolism & Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.0	6.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lysosomal enzymes are synthesized in human fibroblasts as precursors of higher molecular weight and slowly trimmed to the size of tissue enzymes. The enzyme responsible for the limited proteolysis of β-hexosaminidase appears to be an acid protease, activated by thiols and inhibited by leupeptin, similar to but not identical with cathepsin B. The action of the fibroblast protease can be mimicked by trypsin and chymotrypsin. In I-Cell disease, the trimming does not occur, because the hydrolytic enzymes fail to reach lysosomes. The structure and function of the extension peptide(s) are under investigation.

Project Description:

Objectives:

To study normal lysosomal function as well as the biochemical defects in genetic disorders of such function, as in mucopolysaccharidoses and mucolipidoses, and to use the findings for medical assistance to the patients and their families.

Methods Employed:

Conventional biochemical techniques, such as enzyme assays and purification, use of radioisotopes, gel electrophoresis; culture of human diploid fibroblasts from normal individuals and from patients with genetic diseases of lysosomes.

Major Findings:

1. Lysosomal enzymes are synthesized as precursors of higher molecular weight and slowly trimmed to the size of tissue enzymes. A limited proteolysis of β-hexosaminidase appears to be catalyzed by a thiol protease with an acid pH optimum, which is inhibited by leupeptin. A similar limited proteolysis can be achieved by treatment with trypsin and chymotrypsin, though not by a variety of other proteases, including the lysosomal cathepsins B and D. The proteolysis of β-hexosaminidase does not result in any change in catalytic activity.

2. The study of precursor-product relationship has been extended to the enzyme α-L-iduronidase. This enzyme is processed very slowly, several days elapsing between its synthesis as a chain of M_r 75,000 and the complete conversion to M_r 66,000.

3. Proteolytic processing is deficient in intact fibroblasts from I-Cell disease patients. However, that is probably a secondary effect due to the deficiency of a defective recognition marker, which does not allow enzymes to be translocated to lysosomes. Cell-free extracts of I-Cell fibroblasts have the acid protease activity that cleaves β-hexosaminidase.

4. A role for arginine residues in binding of lysosomal enzymes to their receptor has been demonstrated. Iduronidase treated with butanedione, an arginine-specific reagent, has markedly reduced binding to fibroblast membranes, though it retains full catalytic activity.

5. The fate of iduronate sulfatase administered to patients with Hunter syndrome has been monitored. Patients are given plasma (which is rich in iduronate sulfatase) by plasmpheresis, the father or other relative serving as donor. The clinical procedures and evaluations are performed at Johns Hopkins Hospital and the John Kennedy Institute in Baltimore. Iduronate sulfatase persists in the recipients' plasma with a $t_{\frac{1}{2}}$ of about

20 hr. This has not changed even after repeated monthly exchanges. The enzyme is not excreted in urine; whether it is taken up by tissues or simply becomes inactive is not known.

5. A carbohydrate synthesis, which is expected to lead to the production of L-iduronate 2-sulfate derivatives, has been initiated by Dr. B. Weissmann. The ultimate product is for use in enzyme assays, to simplify the screening for Hunter patients and heterozygotes. To date some model compounds have been prepared.

Significance to Biomedical Research and the Program of the Institute:

This project, begun in 1967, has led to the elucidation of biochemical defects and to the development of precise diagnostic methods for a major class of genetic diseases. The discovery that lysosomal enzymes are synthesized in precursor forms has implications for the interpretation of enzyme assays and enzyme purification; for enzyme replacement in genetic disease; for understanding the origin of destructive hydrolytic enzymes in pathological conditions such as rheumatoid arthritis; and for basic understanding of the mechanisms by which cells insure the correct localization of enzymes.

Proposed Course:

1. The structure and function of the extension polypeptides on lysosomal enzymes will be investigated. Is the peptide at the amino or the carboxyl terminus (or both)? Does it function in the recognition of the enzyme by its receptor, along with the mannose 6-phosphate recognition marker?

2. The cell-free synthesis of β-hexosaminidase will be attempted, using messenger RNA from human fibroblasts. This approach is prompted in part by difficulties encountered in studying the phosphorylation of the enzyme with model glycopeptides; we will attempt to use microsomes and their endogenous cofactors (supplemented as necessary) to elucidate the biosynthesis of the phosphorylated oligosaccharide recognition marker on nascent polypeptide chains.

3. The metabolism of the lysosomal enzyme receptor will be studied by techniques similar to those used for the enzymes themselves. How is the receptor incorporated into cell membranes? How is it recycled? This approach will require the production of antibody to the receptor.

4. If the plasma exchange trials show any therapeutic effect, we will attempt to prepare a concentrate of the enzyme from plasma fractions. The preparations will be made in collaboration with the Red Cross Blood Research Laboratory, and will make use of their current separation procedures for medically useful fractions.

Publications:

1. Hasilik, A., and Neufeld, E. F.: Biosynthesis of lysosomal enzymes in fibroblasts. Synthesis as precursors of higher molecular weight. J. Biol. Chem. 255, 4937-4945, 1980.

2. Hasilik, A., and Neufeld, E. F.: Biosynthesis of lysosomal enzymes in fibroblasts. Phosphorylation of mannose residues. J. Biol. Chem. 255, 4937-4935, 1980.

3. Sando, G. N., and Karson, E. M. p-Isothiocyantophenyl 6-phospho-α-D-mannopyranoside coupled to albumin: a model compound recognized by the fibroblast lysosomal enzyme uptake system. I. Chemical synthesis and characterization. Biochemistry, in press.

4. Karson, E. M., Neufeld, E. F. and Sando, G. N.: p-Isothiocyanatophenyl 6-phospho-α-D-mannopyranoside coupled to albumin: a model compound recognized by the fibroblast lysosomal enzyme uptake system. II. Biological properties. Biochemistry, in press.

5. Rome, L. H., and Miller, J.: Butanedione treatment reduces receptor binding of a lysosomal enzyme to cells and membranes. Biochem. Biophys. Res. Commun. 92, 986-993, 1980.

6. DiNatale, P. and Neufeld, E. F.: The biochemical diagnosis of mucopolysaccharidoses, mucolipidoses and related disorders. In Berra, B., Balduini, C., diDonato, S., and Tettamanti, G. (Eds.): Perspectives in Inherited Metabolic Diseases. Milan, Edioermes, 1979, Vol. 2, pp. 113-123.

7. Neufeld, E. F. and Ashwell, G.: Carbohydrate recognition systems for receptor-mediated pinocytosis. In Lennarz, W. (Ed.): Biochemistry of Proteoglycans and Glycoproteins, New York, Plenum Press, 1980, pp. 241-266.

8. Neufeld, E. F.: The uptake of enzymes into lysosomes: an overview. In Desnick, R. J. (Ed.): Enzyme Therapy in Genetic Disease, New York, Alan Liss, 1980, pp. 77-84.

9. Neufeld, E. F.: Recognition and processing of lysosmal enzymes in cultured fibroblasts. In Lowden, A. J., and Callahan, J. (Eds.): Lysosomes and Lysosomal Storage Diseases, New York, Raven Press, 1980 (in press).

10. Neufeld, E. F.: Lessons from Genetic Diseases of Lysosomes. The Harvey Lectures, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52001-03 GBB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Rafael D. Camerini-Otero	Senior Surgeon	GBB NIAMDD
	Michael A. Zasloff	Senior Surgeon	GBB NIAMDD
Other:	Teresa Krakauer	Staff Fellow	GBB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To understand the control of gene expression in higher eukaryotes we must be able to: (a) easily distinguish active genes from inactive genes at the level of the nucleus, and (b) introduce genes with well-defined DNA sequences into cells and observe their expression. Experiments have been performed using both approaches.

This project will terminate this year.

Project Description:

Objectives:

To study the molecular control of gene expression. To use this knowledge to develop new techniques for gene purification.

Methods Employed:

Methods of molecular biology and biochemistry. Recombinant DNA methodology. Techniques of cell culture and somatic cell genetics.

Major Findings:

1. We have been able to introduce genes into mammalian cells. The cells used as recipients have been mouse L cell mutants for biochemically selectable markes: Ltk^- (lacking thymidine kinase, tk), Ltk^- $Aprt^-$ (lacking tk and adenine phosphoribosyl transferase, Aprt), A9 (lacking hypoxanthine-guanine phosphoribosyl transferase, Hgprt) and a line selected by us Ltk^- $Aprt^-$ $Hgprt^-$. So far, the only well-characterized transformants have been obtained with the Ltk^- $Aprt^-$ cells. Genes that have been introduced into this line (which is nonreverting for the tk^- mutation) have been the Herpes simplex virus type I, thymidine kinase gene, and the human and chicken thymidine kinase genes. The frequency of transformation with the purified 3.4 kb BAM H1 fragment containing the Herpes tk gene is about 1 transformant per 25 to 50 picograms per 10^6 cells.

2. We have studied the nucleoprotein structure of the herpes thymidine kinase gene introduced into mouse Ltk^- cells by means of DNA-mediated gene transfer. We have examined, by the technique of Southern blotting, staphylococcal digests of the nuclei from three relatively stable transformants that contain one or less integrated copies of the thymidine kinase gene per haploid genome. Our experiments show that under selection for the active expression of this gene, it is packaged in nucleosomes with a repeat length identical to the average for the host mouse sequences.

Significance to Biomedical Research and the Program of the Institute:

In order to understand normal human development, developmental anomalies and genetic disorders, we must first understand the mechanisms controlling the expression of genes in mammalian cells. The latter is the purpose of much of the work being carried out. Furthermore, the ability to introduce genes into cells might enable us to purify genes that cannot be isolated with the present technology. Many of the genes for known human genetic disorders are of this type.

Publications:

Zasloff, M. A., and Camerini-Otero, R. D.: Limited DNase I nicking as a

probe of gene conformation. Proc. Natl. Acad. Sci. USA 77: 1907-1911, 1980.

Camerini-Otero, R. D., and Zasloff, M. A.: Nucleosomal packaging of the thymidine kinase gene of herpes simplex virus transferred into mouse cells: An actively expressed single-copy gene. Proc. Natl. Acad. Sci. USA, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52002-03 GBB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Role of Cell-Surface Antigens in Development

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Rafael D. Camerini-Otero	Senior Surgeon	GBB NIAMDD
Other:	Teresa Krakauer	Staff Fellow	GBB NIAMDD

COOPERATING UNITS (if any)
Transplantation Biology Section, Immunology Branch, NCI

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The detection and partial characterization of the major histocompatibility antigens encoded by the three H-2 loci, H-2K, H-2D, and H-2L were performed by analyzing immunoprecipitates from detergent-solubilized radiolabeled cells on two-dimensional gels. Each locus was found to code for a population of proteins heterogeneous in both charge and molecular weight. Only part of the heterogeneity was shown to be due to sialylation as demonstrated by shifts in mobility after neuraminidase treatment of antigen preparation. The 2-D gel analysis of the H-2K, H-2D, and H-2L antigens of the d haplotype shows that each constitutes a distinct series of products.

Project Description:

Objectives:

To understand the biochemical mechanisms by which cell-surface antigens are involved in mammalian differentiation and organogenesis.

Methods Employed:

Standard cell biology and biochemical techniques. Two-dimensional gel electrophoresis. Several serologic assays and quantitative immunoprecipitation.

Major Findings:

1. Our results indicate that H-2K, H-2D and H-2L molecules can be resolved into a series of 45,000 m.w. heavy chain spots differing in charge. No evidence for "K-ness", "D-ness" or "L-ness" could be found. That is, K^d was resolvable into a pattern of spots distinctly different from the pattern of both K^k and D^d. Similarly, D^d was readily distinguishable from L^d.

2. Patterns for D^d in separate experiments using the same strain as well as in experiments using different strains bearing the same $H\text{-}2^d$ haplotype indicated that these patterns were specific and highly reproducible.

3. Each locus was found to code for a population of proteins heterogeneous in both charge and m.w. The basis for the unique patterns of spots we have observed for each H-2 is not yet clear. The two most likely explanations that we can envision for this heterogeneity are (i) that each spot represents the product of a separate gene in a tightly linked cluster, the sum of which constitutes what has previously been considered as a single H-2 locus; or (ii) the multiplicity of products represents post-translational modifications of a single gene product for each H-2 locus. The post-translational modification cannot be due to unmodified sialic acid. Treatment of the labeled products with neuraminidase led only to a partial reduction of the heterogeneity. Different modes of glycosylation, amidation, deamidation, or phosphorylation are all other possible means by which charge heterogeneity could be introduced. In order to determine unequivocally which of the two explanations mentioned above is responsible for the heterogeneity, it will be necessary to obtain the genes coding for these antigens.

Significance to Biomedical Research and the Program of the Institute:

This project will lead to a better understanding of the structure and function of cell-surface and histocompatibility antigens.

Proposed Course:

To study the structure of the genes coding for the histocompatibility

antigens. To this end, attempts are now under way to isolate these genes.

Publications:

Krakauer, T., Hansen, T. H., Camerini-Otero, R. D., and Sachs, D. H.: Analysis of the heterogeneity of the mouse H-2K, D and L gene products. J. Immunology 124: 2149-2156, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 52003-02 GBB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure of the Methionine Initiator tRNA Genes in the Human Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael A. Zasloff	Senior Surgeon	GBB NIAMDD
Other:	Tomas Santos	Visiting Fellow	GBB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.5	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have isolated four cloned chromosomal segments from a recombinant phage library of human fetal liver DNA, each of which contains a single $tRNA_i^{met}$ gene. Several novel methods have been developed to study the organization and nature of sequences surrounding each of the $tRNA_i^{met}$ genes in the different chromosomal neighborhoods in which the $tRNA_i^{met}$ gene is embedded.

Project Description:

Objectives:

1. To determine the organization of the $tRNA_i^{met}$ genes in the human genome.

2. To understand the mechanism by which members of the $tRNA_i^{met}$ family associate in the human genome.

3. To understand features of genome organization which relate to the expression of the tRNA gene.

Methods Employed:

All current methods in nucleic acid research including DNA sequencing, fragment cloning, filter hybridization procedures.

Major Findings:

1. The human genome contains at least 11 different copies of the $tRNA_i^{met}$ gene, dispersed over large chromosomal distances.

2. The organization of these genes shows no rearrangements or change in number when embryonic and post-embryonic tissues are compared.

3. Four recombinant phages have been isolated from a library of fetal liver DNA. Each phage contains a chromosomal insert between 11-18 kb, and bears a single tRNA gene.

4. A method called reiteration frequency mapping has been developed to determine the location and genomic reiteration of repetitive sequences within cloned fragments. We have found that each clone has a distinctive fingerprint or repetitive element organization, with no obvious common pattern related to the pressume of the tRNA gene.

5. Restriction endonuclease mapping has revealed that two chromosomal loci bearing a $tRNA_i^{met}$ gene show little similarity. Several 300 base pair elements form duplex structures on heteroduplex studies as analyzed by electron microscopy, corresponding in location to sequences in each clone shown to contain repetition elements.

6. The $tRNA_i^{met}$ gene has been sequenced in two of the cloned fragments. Each is colinear with the mature $tRNA_i^{met}$ sequence, without intervening sequences, and a terminal-CCA. One gene differs from the common vertebrate sequence by the presence of a substitution of G by T at position 57, an otherwise invariant nucleotide in eukaryotic tRNAs.

7. Several sequences in each cloned segment have been identified as transcribed in human diploid fibroblasts. A striking relationship between

two repetitive elements and adjacent transcribed sequences has been noted.

8. A locus in which one of the $tRNA_i^{met}$ genes is embedded resembles a genetically unstable locus in yeast, and a putative recombinationally active sequence has been identified.

Significance to Biomedical Research and the Program of the Institute:

The project attempts to understand the molecular basis of specific gene expression in the human genome and the mechanisms responsible for the origin of the $tRNA_i^{met}$ gene in the genome.

Proposed Course:

1. Sequence analysis of the two $tRNA_i^{met}$ genes will continue, extending in both 5' and 3' directions.

2. The functional activity of each $tRNA_i^{met}$ gene will be studied in an *in vitro* transcription system to elucidate features of structure that affect transcriptional activity.

3. Specific nuclear proteins which interact with the $tRNA_i^{met}$ gene and flanking sequences will be assayed in various human tissues.

4. Other $tRNA_i^{met}$-bearing recombinant phages will be selected from our library.

5. A study of $tRNA_i^{met}$ precursors synthesized *in vivo* will be attempted.

6. The distribution of transcribed sequences identical in the 4-cloned chromosomal segments in total RNA isolated from human diploid fibroblasts will be compared with other tissues, to determine the extent of variation in RNA sequence representation over these chromosomal loci in different tissues.

Publication:

Zasloff, M., and Santos, T.: Reiteration frequency mapping: repetitive sequence organization in cloned chromosomal fragments containing the methionine initiator gene. Proc. Natl. Acad. Sci. USA (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52004-02 GBB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Molecular Basis of Cystic Fibrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael A. Zasloff	Senior Surgeon	GBB NIAMDD
	R. Daniel Camerini-Otero	Senior Surgeon	GBB NIAMDD
OTHER:	Carmen Tejedor	Guest Worker	GBB NIAMDD

COOPERATING UNITS (if any)
Section on Enzymes and Cellular Biochemistry, LBM, NIAMDD
Cystic Fibrosis Center, Children's Hospital National Medical Center, Washington, D.C.

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52005-02 GBB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Eukaryotic Gene Expression and Cytoplasmic-Nuclear Exchange

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael A. Zasloff Senior Surgeon GBB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION
NIAMDD, NIH, Bethesda, Maryland 20205

INSTITUTE AND LOCATION

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52006-01 GBB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Pathophysiology of fibrodysplasia ossificans progressiva and therapeutic intervention

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael A. Zasloff Senior Surgeon GBB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fibrodysplasia ossificans progressiva is a heritable disorder of mesenchymal cell differentiation in which heterotopic bone develops in the connective tissue surrounding skeletal muscle. We have attempted to elucidate the basic cellular defect in this condition from studies of fibroblasts in culture, and tissue implants in receptive animal hosts. Several studies have been started to establish a course of effective medical intervention in fibrodysplasia ossificans progressiva (FOP).

Project Description:

Objectives:

1. To understand the cellular basis of heterotopic bone formation in fibrodysplasia ossificans progressiva (FOP).

2. To elucidate the origin of the remarkable association of pathognomonic skeletal deformities in FOP with the observed cellular lesion.

3. To establish an appropriate course of treatment for affected individuals.

4. To define appropriate sensitive diagnostic criteria in order to follow the progress of the condition.

Methods Employed:

Tissue culture methodology; implantation of human tissue into athymic mice; techniques of diagnostic imaging including computerized axial tomography and bone scanning; routine clinical diagnostic procedures; thermography.

Major Findings:

1. The skeletal deformities in patients with FOP were shown to fall into a pattern suggesting an embryological origin. The hand malformations generally correlate in degree with the extent of foot deformity, and are never observed as an isolated anomaly. We have dated the period of perturbation in limb development in the affected fetus between days 43 to 44 post-conception.

2. A review of the medical literature dealing with skeletal deformities of the limbs has revealed that congenital bilateral hallux valgus, an anomaly associated with FOP, is itself a very rare condition. We have therefore suggested that all children born with this form of digital malformation be monitored closely for the presence of heterotopic bone, and that all unnecessary deep muscle trauma, such as surgical procedures or injections, be avoided. In addition, we have suggested that fetoscopic examination of fetal toes should provide a viable course to prenatal diagnosis in this condition.

3. An 11-year old female with a malignant form of FOP was treated with β-amino proprionitrile (β-APN) over a 3-week period. The rationale behind the use of this agent rested on its inhibition of collagen crosslinking, a critical step in the maturation of bone matrix. In this single trial no drug effect could be appreciated, ossification of a biopsy site proceeding unabated in the presence of significant blood levels of β-APN.

4. Fibroblasts grown from a full thickness biopsy of tissue from above the lateral rectus muscle group of an affected child displayed no abnormal behavior in tissue culture with respect to mineral accumulation, cellular morphology, growth characteristics. Immunofluorescent staining on the tissue specimen, which was removed from an unaffected site, revealed no type II collagen, a marker of chondroblastic tissue. The muscle site on the patient, however, developed a nidus of heterotopic bone within 3 weeks of the biopsy, demonstrating the capacity of the area to accumulate bony tissue.

5. About 10^7 fibroblasts which grew from the biopsy, noted in paragraph 4 above, were implanted as a pellet into the posterior thigh of several nude mice, to determine if these cells would differentiate into hard connective tissue in a more complex milieu. However, after about 3 weeks, neither bone nor cartilage was observed in the implant sites.

Significance to Biomedical Research and the Program of the Institute:

The project attempts to elucidate the cellular basis of a fatal inherited condition, and to provide a rational course of diagnosis and therapy.

Proposed Course:

1. Current drug candidates include Vitamin A and the retinoic acid derivatives. Several patients will be treated with high doses of Vitamin A (50,000-100,000 IU/day) to determine if the described inhibitory effects of this drug on mesenchymal cell differentiation, including the formation of cartilage in embryonic and post-fetal development, will alter the formation of heterotopic bone.

2. Attempts to propagate the abnormal cells in FOP will be continued. The use of skeletal muscle feeder layers, serum supplementation, and manipulation of growth factors will be considered.

Publications:

Schroeder, H., and Zasloff, M. A.: The spectrum of hand and foot malformations in fibrodysplasia ossificans progressiva. Johns Hopkins Medical Journal (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52007-01 GBB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Pseudomonas Infection in Cystic Fibrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael A. Zasloff	Senior Surgeon	GBB NIAMDD
Other:	Carmen Tejedor	Guest Worker	GBB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanism responsible for the colonization of the respiratory tracts of patients with cystic fibrosis by mucoid strains of *Pseudomonas aeruginosa* is being studied. These studies suggest that lytic bacteriophage and several aeruginocins exert selective pressure on the population of *Pseudomonas aeruginosa* in the cystic fibrosis airways, due to the relative resistance of the mucoid strains to phage or aeruginocin-induced bacterial killing.

PHS-6040

Project Description:

Objectives:

To understand the mechanism responsible for the colonization of the bronchopulmonary tree of the patient with cystic fibrosis by mucoid strains of *Pseudomonas aeruginosa*.

Methods Employed:

1. Growth and cultivation of pseudomonads; phage typing of pathological specimens; quantitative phage adsorption assays; isolation of bacterial mutants; characterization of bacteriophage and aeruginocins by electron microscopy, antisera inactivation, buoyant density and bacterial sensitivity spectra.

Major Findings:

1. Stable mucoid variants have been isolated from the bronchopulmonary tract of patients with cystic fibrosis, retaining the characteristic genotype in vitro.

2. The mucoid genotype has been shown to be relatively resistant to killing by all lytic and lysogenic pseudomonas phages tested relative to the non-mucoid variant.

Significance to Biomedical Research and the Program of the Institute:

These studies should extend our knowledge not only of the biological basis of the peculiar and almost pathognomonic colonization of the bronchopulmonary tree of cystic fibrosis patients with a severely pathogenic pseudomonoas strain, but also elucidate several complex ecological interrelationships of pseudomonads and the ubiquitous bacteriophage with which they coexist.

Proposed Course:

1. We will attempt to isolate and characterize bacteriophage from the broncopulmonary secretions of patients with cystic fibrosis.

2. We will continue to study the phage-bacterial interactions which are responsible for the relative resistance to phage injection observed for mucoid strains compared with their non-mucoid variants.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53008-01 GBB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Gene Expression and Human Genetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Rafael D. Camerini-Otero	Senior Surgeon	GBB NIAMDD
Other:	Teresa Krakauer	Staff Fellow	GBB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	0.75	0.50

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to understand the control of gene expression in higher eukaryotes, we are studying how exogenous DNA (genes) are integrated and expressed in mouse cells.

We are investigating the process by which the DNA is integrated. Among the many questions being asked are: (1) what is the DNA into which the first integration occurs; (2) are there specific sequences that promote this recombination; and (3) does the integrated DNA have a mutagenic (or carcinogenic) effect on the host cell.

We are also analyzing the expression of the herpes thymidine kinase gene transferred into mouse L cells. In some cases the phenotype appears to be quickly reverting from thymidine kinase positive (tk^+) to tk^- and back to tk^+.

DNA-mediated gene transfer is also being used to purify selected genes of particular biomedical interest.

Project Description:

Objectives:

To study the molecular control of gene expression in higher eukaryotes and to use this knowledge to develop new techniques for gene purification.

Methods Employed:

Methods of molecular biology and biochemistry. Recombinant DNA methodology and DNA-mediated gene transfer. Techniques of cell culture and somatic cell genetics.

Major Findings:

1. When the herpes thymidine kinase gene is transferred into mouse Ltk^- cells (mouse L cells lacking thymidine kinase), tk^+ transformants are obtained. The DNA from these transformants has been studied by the technique of Southern blotting. In all cases studied the herpes tk gene has recombined with additional DNA.

2. The phenotypic stability of several of these transformants has been studied. In all cases studied, the loss of the tk^+ phenotype was less than 3% per generation. In at least one well-studied case this loss was not accompanied by a genotypic loss of the tk gene. Furthermore, in this case, where close to 100% of the cells appear to have a tk^- phenotype when grown in 5-bromo-2'-deoxyuridine-containing medium, they can quickly revert to a tk^+ phenotype when transferred to HAT medium. The nature of this quick switching of phenotype is being studied.

3. Preliminary results indicate that a large fraction (perhaps as much as 0.1%) of a genome equivalent of foreign DNA enters a transformant. Furthermore, the herpes thymidine kinase gene in transformants is flanked by exogenous DNA sequences. Thus, it appears that the first integration site is into other transferred sequences. Whether this complex is then in turn integrated into host sequences is being investigated.

Significance to Biomedical Research and the Program of the Institute:

In order to understand normal human development, developmental anomalies and genetic disorders, we must first understand the mechanisms controlling the expression of genes in mammalian cells. The latter is the purpose of much of the work being carried out. Furthermore, the ability to introduce genes into cells might enable us to purify genes that cannot be isolated with the present technology. Many of the genes for known human genetic disorders are of this type.

Proposed Course:

1. To modify genes in vitro and then introduce these into cells.

The objective is to study how changes in the DNA affect the expression of the gene and of nearby sequences.

2. To investigate what effect different sequences added to the genes carrying the selectable marker have on transformation.

3. To study whether DNA transferred into a mammalian cell is a mutagen and/or carcinogen.

4. To devise a scheme using DNA-mediated gene transfer to purify genes that code for non-selectable functions. Genes of special interest are those coding for histocompatibility antigens and lysosomal enzymes.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 52009 -01 GBB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chinese Hamster Ovary Cell Mutants with Altered Lysosomal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: April R. Robbins Staff Fellow GBB NIAMDD

COOPERATING UNITS (if any)

Section on Biophysical Chemistry, LNC, NIMH
Sharon S. Krag, Biochemistry Dept., School of Public Health, Johns Hopkins University, Baltimore, Maryland

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.0	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have isolated mutant CHO cells which are deficient in binding and uptake of exogenously added lysosomal enzymes, due to alterations in the mannose 6-phosphate receptor. These mutants secrete 50% of their newly synthesized lysosomal enzymes into the growth media, about five times more than is secreted by the parent cells. Lysosomal enzymes secreted by these mutants contain the mannose 6-phosphate recognition marker, thus mislocation of the mutants' enzyme would appear to result from defects in the receptor. Biosynthesis of the recognition marker has been studied in human fibroblasts and CHO cells. Lysosomal enzymes of a mutant deficient in glucosylation of oligosaccharide-lipid were shown to contain complex oligosaccharides instead of the phosphorylated species. The phosphorylated oligosaccharide obtained from wild type CHO and normal human fibroblasts has the composition: N-acetylglucosamine$_2$ mannose$_6$; this structure gives rise to at least three oligosaccharides, including the phosphorylated species, on the mature enzyme.

Project Description:

Objectives:

To dissect the steps in lysosomal enzyme biosynthesis, modification and translocation using mutants isolated from cultured cells.

Methods Employed:

Techniques of somatic cell genetics, metabolic labeling, SDS-PAGE electrophoresis, enzymology, and carbohydrate chemistry.

Major Findings:

1. CHO cell mutants with altered mannose 6-phosphate receptors were isolated using ricin covalently coupled to monophosphopentamannose as the selecting agent. Of 11 mutants obtained 5 showed decreased uptake of exogenously added lysosomal enzyme. Two mutants with uptake activity $\leq$10% of the parental level were found to have decreased ability to bind β-galactosidase. These mutants were shown to secrete into the growth medium 5-10 times more newly synthesized β-hexosaminidase and α-L-iduronidase than does the parent; about 50% of the mutants' newly synthesized enzymes were found outside the cells. Extracellular levels of α-L-fucosidase, β-glucuronidase and α-mannosidase activities were also elevated in cultures of the mutants. Both α-L-iduronidase and β-hexosaminidase secreted by the mutants are taken up by human fibroblasts via the mannose 6-phosphate receptor.

2. Glucosylation of oligosaccharide-lipid was shown to be necessary for biosynthesis of the phosphorylated oligosaccharides on lysosomal enzyme. Wild type CHO cells and a mutant deficient in this glucosylation activity were metabolically labeled with [2-^{3}H]-mannose, β-hexosaminidase was purified from both cell types by immunoprecipitation, then glycopeptides and oligosaccharides were analyzed by chromatography and digestion with known hydrolases. Approximately 70% of the oligosaccharides from wild type enzyme are phosphorylated; none of the oligosaccharides from the mutant's enzyme contains phosphate, instead most are of the complex type and contain sialic acid.

The phosphorylated oligosaccharides of β-hexosaminidase isolated from both human fibroblasts and wild type CHO cells have the composition phosphate$_{x=1,2}$mannose$_5$N-acetylglucosamine$_2$. An oligosaccharide-lipid intermediate containing five mannose residues is a major species in CHO cells; however, this oligosaccharide is resistant to endoglycosidase H, while the phosphorylated oligosaccharide is susceptible to that enzyme. In addition, we have isolated from human cells a precursor to the phosphorylated enzyme; >90% of the mannose on this precursor is in an oligosaccharide of structure mannose$_6$N-acetylglucosamine$_2$. This oligosaccharide appears to give rise not only to the phosphorylated species, but also to mannose$_4$N-acetylglucosamine$_2$.

Significance to Biomedical Research and the Program of the Institute:

Up until now mutant cells with altered lysosomal function have been obtained from patients with lysosomal storage diseases. Most of the identified storage disorders reflect defects in the individual lysosomal enzymes. In isolating mutants of cultured cells we are able to design screening and selection procedures such that we can obtain mutants hitherto unavailable, e.g., mutants with altered receptors. These mutants are essential to our understanding of normal lysosomal function.

Proposed Course:

1. The linkages between sugars in the phosphorylated mannose$_5$ oligosaccharide and the mannose$_6$ precursor will be determined.

2. Mutants with pleiotropic deficiencies in lysosomal enzymes will be isolated in order to examine the individual steps involved in glycosylation of those enzymes. Glycosylation of viruses will also be examined in these mutants to determine which steps of the process are specific for lysosomal enzymes.

3. Mutants unable to accumulate exogenously added lysosomal enzymes intracellularly will be isolated and examined for (i) enzyme binding, (ii) enzyme internalization, (iii) fusion of endocytotic vesicles with lysosomes, (iv) release of enzymes to the lysosomes, and (v) receptor recycling.

4. Mutants devoid of all mannose 6-phosphate receptor activity will be isolated in order to determine whether some secondary pathway to the lysosomes is available for newly synthesized lysosomal enzymes.

Publications:

None.

ANNUAL REPORT FOR FISCAL 1980
OFFICE OF THE ASSOCIATE DIRECTOR FOR
EXTRAMURAL ACTIVITIES PROGRAM

The Extramural Activities Program (EAP) and the Office of the Associate Director (OAD-EAP) have responded to a wide variety of activities in FY 80 which reflect both the coordinating role of the OAD-EAP and the increasing number of responsibilities being placed upon it and its four branches; the Grants Management Branch, the Contracts Branch, the Program Evaluation Branch, and the Review Unit. The separate branch reports detail accomplishments, challenges, changes, and new requirements as they pertain to the individual branches. This section refers to those activities emanating from the OAD-EAP and those having impact either across the branches of this cluster or across the extramural clusters and the NIAMDD.

Two new programs are now located organizationally within the OAD-EAP. Short Term Training Awards (T35s) authorized as National Research Service Awards were first awarded in FY80. These awards are being administered out of the OAD-EAP since they are awards crossing cluster lines and cannot be correctly assigned to any of our research programs. Currently, the Institute has nine active awards under this program.

The NIAMDD is completing its first full year of participation in the Division of Research Resources Minority Biomedical Support Program. This program, like the Short Term Training Program, is centralized within the OAD-EAP. Total support awarded to this program in FY80 is approximately $800,000.

Finally, New Investigator Research Award applicants are now contacting the OAD-EAP for information about the NIRA program. Although they are awarded by individual research programs, the NIH has established central contact points within each Institute to focus the program. The OAD-EAP is that contact point for the NIAMDD.

This office has made a unique effort in FY80 on developing policies relating to the Institute review of centers and large grant applications. Through a series of memoranda, discussions with staff, and extensive review and modification of procedures, the OAD-EAP has attempted to define the appropriate role of program staff, review staff, and grants management staff in the review process. This review function continues to be closely monitored by this office to keep it responsive to needs of the Institute and to ensure satisfactory peer level review.

During FY80 further efforts were continued to integrate the Contracts Management Branch into the extramural programs family. This included further procedures development and review of the contracts function. To assure a focus for contracts within the OAD, the Special Assistant to the Associate Director has assumed a coordinating and monitoring function for the contracts process within the Institute.

Another focus being developed within the OAD-EAP is on training programs of the NIAMDD. Since the entire issue of training continues to be a volatile one, it was felt that there should be developed a position whose occupant could speak authoritatively on training policy, procedures, etc. This is

being assumed by the Special Assistant to the Associate Director and, hopefully will soon be fully developed.

An increasing emphasis is being felt on the coordinating function for this office. Increasing demands from the Office of the Director, NIH, the Office of the Director, NIAMDD, and the program clusters have resulted in this office being used as a focus for collecting and disseminating information to and from the different groups with whom it coordinates Institute functions.

Several problems continue to affect the ability of this office and the EAP to respond effectively to Institute needs. Inadequate staffing continues to affect our performance in spite of the additional personnel realized in FY80. These additions will be of great assistance when trained and fully productive. Nevertheless, EAP still faces heavy use of overtime and "borrowed" personnel to function properly.

Space is becomming an increasingly more serious problem. Currently, summer employees are being assigned desks which are unoccupied for a day at a time while regular employees are absent. Both the lack of desks and space have emerged as real problems. Employees in some cases have been forced into space borrowed from another Institute. The Review Unit cannot establish its own file system for lack of space, and an entirely new filing system is being proposed for the Operations Section to allow them more room for working on grant files.

A difficult and persistent problem has been the inability of the Administrative Office to provide the administrative services necessary for the cluster to function smoothly. Because of chronic under staffing and a snowfalling workload, particularly the administration of the Chairman's Grant, this office cannot begin to supply all the functions which normally come out of the Administrative Office.

Program Evaluation Branch

The Branch provides information services to administrative and scientific officials regarding NIAMDD programs, especially the extramural programs. These services include but are not limited to scientific analysis and evaluation of ongoing programs and activities; maintaining a Management Information System (MIS); preparing reports from its data base and from those of other MIS's; maintaining the NIAMDD scientific thesaurus and scientifically indexing grant applications assigned to the Institute. In this fiscal year(1980) we will index 4585 applications.

This year there has been an increase in productivity in PEB due in part to modifications in the MIS and the streamlining of operating procedures associated with its maintenance, and in part to the exceptional hard work and team work of Branch staff. As in the past, each Technical Information Specialist is assigned to a Cluster. In 1978 less then 40 special reports and analyses were prepared, in 1979 about 100 were done, and this year over 200 will be completed. These totals do not include reports and responses for program and grants management staff of a routine or recurring nature.

PEB reported and/or coordinated responses concerning Institute's activities during FY 80, for several trans-NIH and national biomedical issues involving research on diseases and in scientific fields within the mission of NIAMDD. Many of these reports and analyses could not have been completed without the professional and technical expertise of two staff members in the Data Section; Mrs. Clementine Whitaker, the Computer Systems Analyst and Mr. Harvey Balderson, the Computer Programmer. They provided special computer programs to gather data for these responses and interfaced between the 'computer' and staff, avoiding collection of garbage.

Some examples of the analyses and responses carried out during the past year are summarized below, followed by the staff member's name who was responsible for its completion:

- The Federal Inventory of Heart, Blood Vessel, and Blood Disorders; a report of projects with a direct relationship to one or more of the NHLBI's twenty national programs. In formulating the Institute's contribution to the inventory, both the CRISP and the NIAMDD scientific information systems were utilized to select projects that met the criteria of the NHLBI for inclusion in the inventory. Over 1000 NIAMDD supported projects were analyzed and 237 were designated as related to the NHLBI national programs. These projects represent an investment of 17.8 million dollars by NIAMDD. (Mr. Robert Petinga)

- The Federal Inventory of Population Research; a report of federally supported projects with relevance to the reproductive process, reproductive endocrinology, contraceptives, fertility/sterility, and reproductive behavior. The Institute's contribution to the inventory was formulated using information in the NIAMDD scientific information system. Over 300 NIAMDD supported projects were evaluated using criteria provided by the NICHD. Fifty nine (59) of these projects worth 4.9 million dollars were included in the inventory. (Mr. Robert Petinga)

- Primary Disease Prevention Inventory, FY 1979; following the guidelines from the OD, NIH 63 projects, funded by NIAMDD for 7.9 million dollars, including all Urolithiasis Centers were reported. (Ms. Linda Stalvey) The breakdown for FY 79 funding is:
  46 Research and Center grants for 6.6 million dollars.
  15 Intramural projects for 1.0 million dollars.
  2 Contracts for 0.3 million dollars.

- NIH Inventory of Clinical Trials, FY 1979; a total of 67 Clinical Trials supported by the NIAMDD were reported by program staff, for 6.9 million dollars, an increase over FY 1978 of 16 trials but for about the same dollars (7.0 million). These trials are being conducted in 31 research grants, 15 contracts, 2 interagency agreements and 13 intramural projects for 20 man years, (Miss Lois Reed)

- Several reviews of the Nutrition and Nutrition Related research have been prepared for the General Accounting Office at the request of the Senate Subcommittee on Nutrition; and for the NIH Nutrition Coordinating Committee. The GAO request was for data on competing Nutrition grant applications (R01's) and how they fared through peer review and funding. Together, with program and GAO staff, we defined Nutrition, and surveyed 8 Council rounds from Oct. 1977 through Jan. 1980. A total of 487 NIAMDD applications fit the Nutrition definition, of these 104 were disapproved, 383 were approved, and 173 were funded for 12.2 million dollars. The priority range of the funded grants was 107 to 258. (Mr. Harry Free)

- At the request of the Human Growth Foundation and utilizing PEB's scientific indexing system, and analysis capabilities we reported the support of 16 NIAMDD research grants for 1.1 million dollars funded in FY 79 with primary emphasis on growth hormone research. Also reported were 71 grants for 7.2 million dollars in which some growth hormone research was being carried out. (Ms Linda Stalvey)

- NIH Research in Kidney Diseases for FY 1979; determined the appropriate indexing terms for inclusion of all kidney diseases and related disorders, and from the CRISP system, selected and analyzed abstracts of projects funded by NIH, for relevance to the kidney and urology fields. Over 1000 abstracts were selected to be used in updating the Data Book of 1976 on "NIH Research in Kidney and Urinary Tract Diseases". A similar procedure was undertaken to determine NIH support of research on End Stage Renal Disease. (Mr. John Palmer)

An extensive update and edit of the NIAMDD scientific thesaurus has recently been completed. In general, new biomedical indexing terms were added and others modified to better describe advances or changes in scientific research, specifically as they affect research supported by NIAMDD. This thesaurus will be the major component of PEB's Users Guide which, it is anticipated, will be completed this fiscal year and distributed to Institute staff.

Recruiting has begun for a Computer Programmer and it is hoped that selection of a candidate will be made by summer's end. Filling this critical position will allow the Branch to maintain its current level of productivity.

Reporting requirements for Contract data are increasing continually. Data Section staff have written or modified more then 10 computer programs that display contract information in various formats to meet the needs of users.

In summary, we did not accomplish everything planned for FY 1980, due to limited resources and increased responsibilities and reporting requirements. However, we did complete essential tasks; streamline internal operating procedures; cease duplication with the IMPAC system; eliminate working all but the most essential documents; and increase the credibility of the data in the MIS. As our resources increase or as technological advances allow, we will improve ongoing services and add new ones.

CONTRACTS MANAGEMENT BRANCH

Although annual obligations for this year are not expected to deviate appreciably from the prior year, overall workload has increased because Freedom of Information (FOI) inquiries and other administrative/statistical reporting requirements have accelerated. One noteworthy example is the Secretary's three phase approach to procurement planning and control of year-end spending. Salient features include:

- Implementation from the top down, with emphasis on senior program managers having the task of early identification and definition of contract needs;
- Concurrent coordination with the contract office to agree upon feasible goals by jointly completing the HHS Form 592;
- Submission to HHS of planning schedule documents which chart both the distribution of requests for contracts and targeted award dates;
- Maintenance of internal records to be made available to review teams from the Assistant Secretary for Management and Budget (ASMB) and Inspector General (IG).

NIAMDD has been under sporadic audit surveillance throughout the year, with several visits from both the GAO and IG. It does not appear that the future will be any different.

Training of personnel to meet certification requirements was intensified. Except for one new employee, everyone has applied for an appropriate level of certification. Since some applications may be disapproved, more training may be needed.

Efficiency continues to be hampered by inadequate accomodations. Both productivity and quality could be increased by improved working conditions.

Our competitive/noncompetitive award ratio is still being closely monitored by the Department. New noncompetitive contract awards can hardly be considered because of strict review by higher echelons of concurrence and approval. Even legitimate continuations and/or renewals are being closely scrutinized with the intent of subjecting at least some portion to competition.

All closeout actions are being closely tracked - especially submission of final reports - but the overall closeout problem is currently under control.

In conclusion, many of the former issues that surrounded cooperative agreements remain; to date no substantial impact has been experienced. Congressional socioeconomic mandates have usurped the traditional legal concept of contract award instruments, with consequent implementation bottlenecks. Finally, the single most pervasive Departmental objective is the exercise of more control over both contract awards and expenditures thereunder. Therefore, partially attributable to media exposes, accountability by Federal and contractor

personnel has become the keystone for proliferation of increasingly rigorous and time consuming regulations.

GRANTS MANAGEMENT BRANCH ANNUAL REPORT

Due to the necessity to improve the efficiency of the branch in the face of personnel ceilings, we have this year initiated a procedure of utilizing facsimile signatures on award notices. That is, both the Grants Management Officer's as well as the Program Directors' signatures are affixed to awards using a signature stamp. While this procedure has certain negative aspects such as the Program Director being unable to personally review the award before issuance, the positive aspect is that this procedure saves approximately 2 days of processing time.

The implementation during this fiscal year of changes in the level of stipends for NRSA awards has caused considerable increased work for the branch. This is especially true because of the fact that the effective date for the increase was July 1, 1980, rather than the start of the fiscal year. Because of this, it has been very difficult to estimate the increased NRSA budget requirements caused by these stipend adjustments. The workload for NRSA's has been increased substantially (for FY 1980 only) due to the need to revise or supplement many of our training and fellowship awards.

From an overall budget standpoint, the Institute and GMB have been hampered by decreased flexibility in the allocation of extramural funds to program areas and mechanisms of support. While in some respects the specific problems with insufficient RCDA funds and the need for reprogramming is not directly related to GMB's budget execution function , it has had a serious affect on our ability to fund awards. Further, it has increased the complexity of our award decision process in that we must be cognizant of applications being classified as R10's, R09's, R13's and K's as well as their relative priority score standing.

In the area of personnel, several important developments occurred during this year. First, we were allowed to recruit for a Deputy Chief of the branch when that position became vacant during the year. The new person, who was recruited from outside the Institute, is now on-board and functioning. In addition, we were allowed to fill 2 clerical positions, one in the central office of the branch and one in the award unit. Both of these recruitments reflect the return of slots that were lost to the branch over the last several years. We feel that this indicates a strong recognition within the Institute that the Grants Management Branch requires additional personnel in order to keep pace with the workload. We also became involved in a formal training program that allowed Grants Technical Assistants within the Institute to apply for a Grants Management Specialist trainee position within the branch. From among over 10 highly qualified candidates, we made our selection. This individual is now in training to become a fully functional Specialist.

While there have been significant positive developments in personnel, there remain equally significant unsolved personnel problems. With regard to our clerical staff, we are still confronted by confusing, conflicting or non-existent classification standards for Grants Assistants and Grants Clerks. In 2 instances, we have been prevented from promoting or reassigning clerical people due to this lack of standards. While this is a problem faced by every office employing such personnel, it is especially severe for Grants Management because of lowered morale in the face of increasing workloads.

Again this year we are striving to reach the Department's goal of obligating at least 70% of grant funds by the end of the 3rd quarter (June 30, 1980) of the fiscal year. At best, there is only a remote chance of our achieving this goal. The best prediction now is that we may come close to 65%. At the same time however, it should be recognized that a major effort was undertaken within the branch to achieve this goal. For instance, all of the Diabetes Research and Training Centers and most of the Kidney SCOR grants with September 1, 1980, start dates were encumbered prior to June 30. It is important to note that even in attaining the 65% level massive amounts of overtime have been needed. Indeed, this requirement for overtime to be performed by clerical personnel of our Award Unit has been the normal mode of operation for over a year. It is only through these efforts, as well as overtime performed for us by clerical help from certain program areas, that allow us to be this close to our goal. Therefore, it is imperative that additional full-time slots be allocated to the branch.

For the Operations or file room section of the branch, a major problem continues to be a shortage of both floor space as well as shelving space for the storage of official grant files. However, a recent renovation of the file room has made the space more conducive to the needs of the personnel. In addition, we are examining the possibility of procuring movable shelving to replace existing stationary units. We believe that this will almost double our shelf space while utilizing the same amount of floor space. This will allow more efficient use of our limited office area.

For the rest of the branch, lack of space is also a continuing problem. However, on the positive side, a renovation of our space on the 6th floor has been completed. This has enabled us to assign appropriate separate office spaces to each of our Grants Management Specialists. This provides needed privacy to these individuals. Last year, we reported that 2 of our personnel were located in space on the 4th floor while the remaining members of the branch were on the 6th floor. Further, we expressed concern about our ability to maintain proper supervision in this remote location. That problem continues and has been worsened by the fact that NHLBI has reclaimed space that the supervisor of our file room had occupied. Therefore, that individual has also been shifted to the 4th floor resulting in still more supervisory problems for the branch.

ANNUAL REPORT FOR FISCAL 1980
REVIEW UNIT, EXTRAMURAL ACTIVITIES PROGRAM
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

The Review Unit is now responsible for the technical merit review of applications for training grants, cooperative clinical studies, clinical investigator awards, program projects, core center grants, specialized center grants, comprehensive center grants and for contracts. The applications for cooperative clinical studies and for clinical investigator awards reflect new responsibilities for the Review Unit.

Although the total number of applications under review has been quite large at times, our present review staff has been able to provide quality merit reviews through use of the flexibility inherent in the ad hoc review mechanism. Evaluations by our ad hoc consultants of grant proposals reflected in Table 1 requesting a total of 36.34 million dollars resulted in recommendations for funding of approximately half the funds requested, i.e., 15.57 million dollars. Evaluations of contract proposals requesting a total of 37.72 million dollars resulted in recommendations for possible funding of 13.53 million dollars.

TABLE 1. NUMBER OF PROPOSALS REVIEWED BY REVIEW UNIT FOR FY 1980

Activity Code*	COUNCIL Aug. '79 Oct. '79	Jan. '80	May '80	Total
P01	5	5	3	13
T32	18	23	41	82
P30, 50, 60	7	5	19	31
N01	72	3	11	86
R10	2	-	2	4
TOTAL	104	36	76	216

*P01 = Program Projects, T32 = Institutional Fellowship Applications
P30 = Core Center Applications, P50 = Specialized Centers of Research
P60 = Comprehensive Centers, N01 = Contract Proposals. Those reviewed from the first day of one Council month to that of the next Council month are assigned to the latter Council.
R10 = Cooperative Clinical Research

In response to concerns expressed by program staffs and members of the scientific community, steps are being taken to establish standing committees to undertake the technical merit review of applications for institutional fellowships, clinical investigator awards, program projects and centers. The organization of two review groups for institutional fellowships and clinical investigator awards is well underway. A "Special Projects Review Committee" for review of centers and program projects is pending approval for a charter. However, similar requests for the past few years have been without

success, and in fact, the only chartered committee within NIAMDD which functioned for contract review, was not approved for renewal this past year when its charter terminated. It is not clear that the use of standing committees as now contemplated and being proposed will be completely feasible without additional resources. Because of this, a highly conservative approach is being used to phase the present ad hoc committee operation into a "standing" committee operation.

The staff of the Review Unit is now comprised of 12 people, including six professional members. All allocated positions are filled. The Review Unit has been restructured from three sections to two sections in order to improve the management of the technical merit review of the broad spectrum of applications. To further enhance review activities, both in terms of quality and a more effective use of staff, a centralized "working review" file system needs to be created. However, the constraints imposed by the available space to the Review Unit prevents implementation at this time.

The constraints of time imposed by Council rounds, the magnitude and variability of the workload, and staffing limitations require that a grants clerk be available to substitute for one of those in the two sections should illness or change in employment occur. Further, such a substitution should occur with a knowledgable grants clerk familiar with the operation of the Review Unit. This can readily be accomplished through recruitment at a GS-3 or 4 level for a clerk typist who would be assigned to the office of the Chief of the Review Unit to function in this role, and to also assume the responsibilities of "timekeeper" and/or disbursing pay check agent. The assumption of these and other duties by this grants clerk would permit a greater role to be played by the grants technical assistant presently in this office for assuring quality document preparation and timely document flow by the grants assistants within the Review Unit. An additional position needs to be allocated to the Review Unit for this purpose.

A Savin model copy machine has been installed in close proximity to the Review Unit. This should save considerable time and effort of staff who have been traveling several floors constantly to make a few copies of one or two pages of documents as review packages are developed for consultants.

Efforts have been made to encourage staff to take advantage of training workshop opportunities. As a result, six of the staff members have taken one or more formal courses offered this past year.

ANNUAL REPORT

DIABETES, ENDOCRINOLOGY, AND METABOLIC DISEASES CLUSTER

OFFICE OF THE ASSOCIATE DIRECTOR

The Associate Director plans and directs programs of research grants and contracts, training and fellowship awards, and special programs and activities within the Diabetes, Endocrinology, and Metabolic Diseases Cluster. The Associate Director is responsible not only for strengthening, integrating, and coordinating activities within these areas but also for developing and initiating new programs to meet the needs of the scientific community.

In the area of diabetes, the Associate Director, acting through the NIH Diabetes Coordinating Committee, has primary responsibility for the management of all trans-NIH activities relating to diabetes mellitus, assuring better coordination, a sharper focus for program activity, and accountability in diabetes among Institutes.

The Associate Director also serves as Chairman of the Inter-Agency Diabetes Mellitus Coordinating Committee, whose membership includes representatives from all federal agencies with an interest in diabetes mellitus. He plays a lead role as liaison to voluntary health groups and scientific societies concerned with diabetes, endocrinology, and metabolic diseases. Thus, he is frequently and intimately involved with the activities of such groups as the American Diabetes Association, the Juvenile Diabetes Foundation, the Cystic Fibrosis Foundation, and the Endocrine Society.

The Associate Director also directs special activities concerned with diabetes, endocrinology, and metabolism. He is conducting a study of the feasibility and desireability of providing support for research resources such as hormones and other biological products, experimental animals, and specialized equipment within the Institute's Endocrinology and Metabolic Diseases Programs. He is also directing a study of the desireability and feasibility of NIAMDD support for a clinical trial investigating the effect of strict blood glucose control on the development of the vascular complications of diabetes. In this regard plans have changed significantly from those summarized in last year's Annual Report. The multi-center collaborative clinical trial was temporarily postponed in early FY80 in order for the Institute to assess the opportunities presented by recent progress in the development of new automated insulin delivery systems and devices for home monitoring of blood glucose. An ad hoc consultant group of representative experts in the field was convened to review research progress in these areas and to advise the Institute with respect to the potential impact of these new technologies on the proposed trial design. This group, which met formally during the winter and spring of 1980, recommended that the Institute modify its preliminary plans for this trial and include these new technologies. At the present time, the preliminary trial design is being refined in order to help ensure that the critical question of the relationship between control and complications is addressed with the very best research tools the state-of-the-art-can provide.

In addition to his programmatic responsibilities and coordinating activities, the Associate Director carries on his own program of research. His laboratory is investigating the relationship between diabetes and obesity with a particular focus on the adipose cell as a model for the study of insulin's regulation of glucose metabolism.

Diabetes Programs Branch

This office coordinates the activities of the Diabetes Research Program, Diabetes Centers Program, National Diabetes Data Group, and National Diabetes Information Clearinghouse. In addition, it provides an administrative focus for extramural grant and contract support of various special research initiatives and for liaison regarding training, career, and resource development activities in diabetes and related areas of endocrinology and metabolism. It also coordinates support for diabetes-related awards in other NIAMDD programs and for diabetes-related workshops, conferences, and symposia sponsored by the Institute. The highlights of these activities are summarized, as appropriate, in the following sections of this report. Although not detailed below, the staff of the Diabetes Programs Branch also serves as an information resource to the scientific community, lay public, and other federal, state, and private organizations with respect to the full spectrum of national diabetes research, training, and information-transfer activities. In addition, the staff also participate in and contribute to a wide variety of NIH-sponsored activities which, directly or indirectly, support the continued development of expanded scientific capabilities both in diabetes and other areas of biomedical research.

Diabetes Research

During the past year, progress has continued in a wide range of scientific disciplines which are fundamental to research on diabetes mellitus and its complications. These studies focus primarily on defining the nature, etiology, epidemiology, and pathogenesis of diabetes and its complications in a long term effort to develop improved medical capabilities for prevention, diagnosis, treatment, and cure of this disease. Within this context, some areas of particular research interest include studies involving islet cell transplantation; insulin delivery systems; the epidemiology of diabetes and its complications; the biosynthesis, secretion, action, interaction, and degredation of insulin and other related hormones; the physiology and pathophysiology of pancreatic islet cells; abnormalities of carbohydrate, lipid, and protein metabolism associated with diabetes and its complications; the possible relationship between various genetic, viral, immunologic, toxicologic, and nutritional parameters and the etiology of diabetes and its complications; and biobehavioral research related to the psychosocial aspects of diabetes. Recent progress in some of these areas is summarized below:

Islet Tranplantation. As noted in last year's Annual Report, grantees of the program developed new techniques which markedly prolong survival of rat islet allografts across a major histocompatibility barrier. These techniques involve both low temperature pre-incubation of donor cells in tissue culture and immunosuppresion of the recipient immediately prior to the transplantation procedure. More recently, these investigators have extended their

studies utilizing this technique and reported successful transplantation of pancreatic islet cells from one animal species (rat) to another (mouse) without immunologic rejection. These investigators have further shown that the transplanted insulin-producing islets could reverse diabetes in the mouse recipients as evidenced by the subsequent maintenance of normal blood glucose levels for more than four months following the experimental procedure.

These animals will be closely observed to determine whether rejection of the transplant will occur at a later time or whether the procedure has, in fact, produced indefinite graft survival.

Although the experiments noted above suggest that immune rejection may not prevent the further development of islet transplantation as a successful therapeutic approach to diabetes, a number of significant problems remain to be addressed before the potential clinical application of these procedures can be established. More specifically, it will be necessary to (1) define further the mechanism of immune rejection and prevent its occurence so that islet cell transplantation can be successfully evaluated in the human; (2) develop improved techniques for selective, mass isolation of beta cells from pancreatic tissue of various large animal species; (3) develop improved procedures for long-term storage of islet cells prior to transplantation; (4) evaluate further the relative advantages of various transplantation sites with regard to the specific survival and functional integrity of beta cells; and (5) assess the impact of successful transplants on plasma glucose control and other indices of metabolic homeostasis in diabetes.

Insulin Delivery Systems. Another promising area of research interest is related to the continued development of improved devices for regulating the delivery of insulin to the diabetic. As noted in last year's Annual Report, further development of miniature "closed-loop" insulin delivery systems is limited at the present time by a number of factors, foremost among which is the lack of a reliable and specific glucose sensor with physical and biological characteristics that would make it appropriate for long-term clinical studies. The Institute recently sponsored a workshop on glucose sensor technology in order to assess the state-of-the-art and to stimulate new and expanded interest in this important area of research. In the meantime, in the absence of a suitable glucose or other metabolite sensor, the Program is providing increased support for the further refinement and evaluation of various "open-loop" devices which essentially consist of an insulin pump and a control circuit that regulate the rate of insulin infusion in a pre-programmed fashion. These devices, which are portable and offer the potential for use in long-term ambulatory studies, are presently being evaluated by a number of research teams both in this country and abroad. One group of investigators has recently reported not only improved control of blood glucose in juvenile-onset diabetics who were treated with chronic subcutaneous insulin infusion from such a device, but also normalization of both plasma lipids (including cholesterol, triglycerides, and free fatty acids) and branched chain amino acids (valine, leucine, and isoleucine). In addition, these investigators have also reported that the exaggerated increments in plasma growth hormone, epinephrine, and norepinephrine which are induced by exercise in conventionally-treated diabetics are reduced to normal within two weeks of pump treatment.

These studies, as well as those by a number of other NIAMDD-supported investigators, suggest that automated insulin delivery systems may offer the potential for a significant improvement in the treatment of insulin-dependent diabetes. However, at the present time, no existing device has been demonstrated to be suitable for long-term insulin administration in other than a research setting. Current treatment of diabetes with a portable insulin-infusion system remains experimental and should be limited to situations involving appropriate research protocols and investigators with necessary expertise. Nevertheless, if advancements in this area continue as they have during the past several years, such devices may facilitate the conduct of a study to definitively assess the effect of plasma glucose control on the development of the chronic complications of diabetes. When available, the results of such a study would represent a major step forward in our understanding of the pathophysiology of diabetic complications and would subsequently determine the future direction of efforts to improve the therapy of diabetes.

Insulin Action. Despite intense research in the area of insulin action at the level of the target tissues, the details of this complex sequence of events is still not completely understood. However, various new insights into membrane receptor dynamics, membrane transduction and transport systems, and intracellular processes have increased our understanding of how insulin regulates cellular glucose metabolism.

The action of insulin is initiated by the interaction of the hormone with specific receptor sites on the cell membrane. In last year's Annual Report, it was suggested that this interaction might generate a signal(s) which activates the various intracellular mechanisms responsible for insulin's biological effects. More recently, it has been reported that a soluble factor(s) is generated in target cells in response to insulin and that this factor(s) is able to modulate various enzyme systems in vitro. Thus, muscle extracts from insulin-treated tissue have been reported to inhibit cyclic AMP-dependent protein kinase and to activate glycogen synthase phospoprotein phosphatase. The active factor(s) which has been partially purified also appears to enhance adipocyte pyruvate dehydrogenase in vitro. In other experiments, a similar factor(s) which enhances pyruvate dehydrogenase activity in isolated mitochondria has been extracted from fat cells. Furthermore, this adipocyte factor has been generated following the direct addition of insulin to isolated plasma membranes. These recent findings suggest that model systems may now be available to study the molecular nature of intracellular mediators in insulin action.

During the past year, another group of grantees has accumulated additional evidence which suggests that a primary defect in diabetes is related to the abnormal membrane transport of Na+ and H+. In this regard, it has been reported that insulin elevates intracellular pH, and that if this action of insulin is blocked, the effect of insulin on glycolysis is also blocked. This suggests that the hypoglycemic action of insulin may be secondary to a primary effect on cation transport.

In other laboratories, work has continued to further define the effects of insulin and various other hormones on carbohydrate and fat metabolism in liver cells. Of particular significance in this regard are studies designed

to determine the molecular basis for reversible regulation of glucose and fat synthesis and utilization in hepatocytes. It now appears that the interconversion of key enzymes between active and inactive forms by the phosphorylation-dephosphorylation mechanism may be of considerable importance in the hormonal control of these metabolic processes.

Insulin Resistance. Another area of significant research interest during the past year has involved studies related to insulin resistance in target tissues and associated defects in insulin activity. It has been reported that a maximal insulin effect in man is achieved at an insulin concentration which does not saturate the total number of insulin receptors, and that maximal glucose transport occurs on isolated adipocytes when only 10% of the receptors are occupied. Therefore, it appears that many cells possess a relatively large number of "spare" insulin receptors. This might suggest that a relatively large decrease in the number of receptors would be required to account for the decrease in insulin action associated with insulin resistance, and thus a post-receptor defect at some rate-limiting step in glucose metabolism has also been proposed as the basis for this phenomenon. With respect to insulin resistance in human obesity, recent evidence seems to suggest that a decrease in the number of insulin receptors and a consequent decrease in sensitivity to prevailing submaximal insulin levels may account for part of the insulin resistance of some patients. In addition, it now appears that many obese patients may demonstrate a combined defect with decreased insulin sensitivity due both to decreased insulin receptors as well as to post-receptor defects. One such post-receptor defect that has been demonstrated in rat adipose cells and which appears to account for at least part of the insulin insensitivity in obese animals is at the level of glucose transport. There is not yet a complete unanimity of opinion regarding the regulation of insulin receptors and the nature of their proposed role in insulin resistance, and further research will be required to fully define the mechanisms for these and related interactions.

Insulin Secretion. In the area of insulin secretion, grantees have recently confirmed that the transduction of secretory information in pancreatic B-cells is associated with transmembrane ion fluxes and their consequent electrical signals. In addition, it has also been demonstrated that myinositol is necessary to maintain the functional integrity of B-cells in tissue culture. These and related studies suggest that although glucose metabolism alone is not sufficient to maintain the insulin-secretory capacity of B-cells, it may subserve the maintenance of essential transmembrane-ionic gradients. Such observations may eventually lead to the development of new types of drugs to treat diabetes.

Another group of investigators have extended studies in the Chinese hamster to further define and characterize the mechanisms of insulin and glucagon release and have recently shown a clear correlation between hormone release and cell membrane depolarization. In addition, they have also observed that increased Ca++ levels can produce hyperpolarization and associated inhibition of insulin secretion.

Recent reports on regulation of the endocrine pancreas through neurohormonal mechanisms have confirmed earlier studies which identified an insulin release

inhibiting factor (IRIF) and glucagon releasing factor (GRF) in the hypothalamus of the rat. It has also been noted that GRF appears to act in the islet cells through a mechanism mediated by adenyl cyclase. Further studies of islet cells in rats bearing ventromedial hypothalamic lesions have shown an altered glucose sensing function of both alpha and beta cells in vivo and in vitro. These responses appear similar to those which characterize the obese hyperglycemic mouse (ob/ob) as well as the hyperinsulinemic adult diabetic. Since the ventromedial hypothalamus is also the site of the "satiety center", these findings are considered to be potentially important because of the relationship between diabetes and obesity.

Insulin Synthesis. The synthesis of insulin, using recombinant DNA techniques, has continued as a very active area of research during the past year. As summarized in last year's Annual Report, a team of researchers have used a copy of the rat proinsulin gene for induction of proinsulin synthesis in E. coli bacteria. These investigators have now extended this process and demonstrated bacterial production of small quantities of biologically active rat insulin, and have identified separate clones containing either the rat insulin I gene or the rat insulin II gene. Another group of investigators has recently sequenced the human insulin gene and compared it to the rat insulin I gene. Related studies have also been reported on mutant forms of insulin which are associated with specific types of diabetes and familial hyperproinsulinemia. In this regard, allelic differences have been identified in the 3' -untranslated region of human insulin mRNA which indicate a form of polymorphism that could affect insulin biosynthesis. These results suggest that under conditons of metabolic stress, the biosynthesis of mutant forms of insulin might occur which in turn could result in diabetes.

Diabetogenic Agents. During the past year, there has also been considerable progress in defining the mechanism of action of several drugs shown to produce a diabetes-like condition in experimental animals. In this regard, it has been demonstrated that the mechanism of action of these drugs involves effects on various oxidation/reduction reactions which are fundamental to cellular metabolism. For example, streptozotocin (SZ) has been shown to decrease pyridine nucleotide levels in isolated islets, to decrease levels of reduced glutathione in erythrocytes, and to deplete levels of superoxide dismutase (SOD) in erythrocytes and in the retina. In as much as SOD appears to provide some amelioration of the diabetogenic effect of SZ, it has been suggested that the latter may be mediated in part by SZ-induced impairment of the ability of the B-cell to withstand oxidant stress. Moreover, studies of humans who have ingested rodencide, a rat poison which is somewhat similar in structure to SZ, demonstrate that this agent exhibits a specific B-cytotoxicity as well as produces both autonomic and peripheral neuropathies. Therefore, cases of rodencide poisoning may represent unique models for acquired insulinopenic diabetes in humans, and suggest the possibility that environmental toxins of related structure might be responsible for inducing certain cases of insulin-deficient diabetes mellitus. Given this as well as the established coupling between electron flow, membrane transport, and the activity of isoenzymes of pyridine-nucleotide-linked

dehydrogenase and other glycolytic enzymes, the mechanism of oxidant action may also provide insights into fundamental aspects of other etiologies of diabetes.

Immunological Factors. As summarized in last year's Annual Report, another area of particular research interest involves the immunologic defects associated with diabetes. A variety of different studies involving the assessment of immunologic status, genetic constitution, and susceptibility to viral infections are now beginning to provide insight into cell-mediated immunologic abnormalities associated with insulin dependent diabetes. Some such abnormalities have now been described and have been localized to the T-lymphocyte and to lymphokine production.

Studies are now in progress by one group of investigators to determine the role of islet cell surface antibodies and to characterize islet cell antigens. Another group of investigators is attempting to define the mechanism by which peptide hormones and neurotransmitters regulate the "vigor" of immunity. Preliminary results from these studies have shown that lymphocyte activation first stimulates a rapid DNA-independent emergence and then subsequent loss of insulin receptors. In addition, the possibility of neuroendocrine regulation of immunity in healthy and diabetic animals is also being explored. Further confirmation of the role that cell-mediated auto-immunity plays in the pathogenesis of diabetes is found in studies of the BB/Wistar rat, a spontaneously diabetic animal model. Injections of rabbit antiserum against rat lymphocytes have reversed hyperglycemia in 36 percent of spontaneously diabetic rats and prevented diabetes in susceptible nondiabetic controls. Another group of grantees has shown that the serum from some insulin-dependent diabetics has a cytotoxic effect on isolated hamster pancreas islets in vitro, which suggests that a complement-mediated process is involved and that the cytotoxic factor is a circulating antibody.

Diabetes Centers Program

Background. This program provides support for both Diabetes-Endocrinology Research (DERC) and Diabetes Research and Training Centers (DRTC). The primary requirement for establishment of either a DERC or a DRTC is a strong base of high-quality, ongoing biomedical research. In addition, DRTCs also have a training/information transfer component which includes training of both medical and allied health professionals, continuing education, model demonstration, and outreach activities. For both types of centers, the grant provides for core facilities (shared resources), pilot and feasibility studies (new initiatives), and limited funds for program enrichment. This funding is intended to strengthen existing programs, increase productivity, and foster the generation of new ideas and innovative research approaches.

While the emphasis in the DERC centers primarily around biomedical research, the fundamental objective of the DRTC is broader, and includes the creation of an environment which fosters and integrates the efforts of basic and clinicial scientists, physicians, allied health personnel, and educators joined together in a cooperative, multidisciplinary approach to the many research and health care problems associated with diabetes and its complications. Therefore, the DRTC program represents not only a concerted effort

to achieve a better understanding of diabetes through biomedical research, but also to improve the delivery of health care to the diabetic patient through training and continuing education programs. Although each center is expected to develop its own independent program in accordance with local needs, interests, and resources, each is also expected to be responsive to national needs, and must be willing to work cooperatively with the NIAMDD and, in the case of DRTCs, with other federally-supported diabetes programs.

To provide a forum for interaction among the centers, the NIAMDD sponsors an annual center meeting in June of each year, in conjunction with the national meeting of the American Diabetes Association. Participants include the principal investigators and key particpants from the centers, the NIAMDD staff, and representatives from other Federal agencies with diabetes programs which interface with center activities. These meetings often lead to new ideas or avenues of approach for cooperation and collaboration which can be explored subsequently by smaller groups throughout the following year.

Presently Funded Centers. During the past year, the program provided support for 3 DERCs and 8 DRTCs as follows: Diabetes Endocrinology Research Centers - University of Pennsylvania (Philadelphia), University of Washington (Seattle), University of Iowa City (Iowa City); Diabetes Research and Training Centers - Albert Einstein College of Medicine (Bronx), Indiana University (Indianapolis), University of Michigan (Ann Arbor), Washington University* (St Louis), University of Chicago* (Chicago), Joslin Diabetes Foundation, Inc. (Boston), Vanderbilt University* (Nashville), University of Virginia* (Charlottesville).

A new mechanism of support has been developed which allows DERCs the opportunity to become DRTCs. A DERC may apply for a modest exploratory grant to develop the resource necessary for initiation of DRTC training and information transfer activities.

Research Activities at the Centers: Funding of research at the centers comes from NIH R01 and P01 (or equivalent) grants to center investigators. However, the concept of shared biomedical resources provided by the center funding allows for enhanced productivity and increased multidisciplinary efforts. The following information may serve to illustrate this point.

At the Washington University, St. Louis, DRTC a variety of studies related to transplantation of pancreatic islet cells are in progress in several laboratories from various sections of the medical school representing different disciplines. Last year center participants reported on the development of techniques to permit successful islet transplants between animals of different genetic strains. Work is being continued on this study which has potentially important implications for the future of islet cell transplantations in human diabetes. Three separate DRTC core facilities, Radioimmunoassay, Morphology, and Tissue Culture, contributed significantly to this work.

* Previously supported as a DERC.

Another example of the multidisciplinary approach comes from the University of Chicago where center participants from several basic and clinical science laboratories have been investigating the structure and function of the pancreatic hormones, insulin and glucagon. During the past year, these investigators reported their studies of the first patient with diabetes caused by a mutation in the insulin gene, resulting in the production of some abnormal insulin. This variant insulin not only is unable to act normally to regulate the body's metabolism of glucose, but also interferes with the action of normal insulin. This research, resulting from the collaborative efforts of several researchers, represents the discovery of a new cause of diabetes in man, and contributes significantly to our understanding of diabetes. Three core resources supported by the DRTC were utilized in this study: Islet and Cell Isolation and Culture, Peptide Purification and Structural Analysis, and Radioimmunoassay.

Pilot and feasibility studies are another aspect of the research component of centers. The center awards provide modest support for three years or less to allow researchers to explore the feasibility of a concept and amass sufficient data to apply for independent funding. This mechanism provides a unique opportunity not only for testing new ideas, but also for young investigators to become involved in diabetes research, and established investigators in other disciplines to apply their expertise in the areas of diabetes.

For example, Indiana University DRTC is supporting a pilot and feasibility study related to neurologic complications of diabetes. This project has been initiated by two young investigators and an established investigator who had previously been working on neurologic disorders unrelated to diabetes. With DRTC support, these investigators are now examining the spinal cord and peripheral nerves in diabetic rats. Their work so far indicates more extensive neurologic damage in the diabetic animals than was originally anticipated. They are also examining whether early intervention by control of blood sugar will prevent this nerve damage. At least two other aspects of the DRTC have contributed to the success of this project: the availability of shared resources provided by the DRTC, and access to experienced diabetologists facilitated by the multidisciplinary DRTC environment.

Through an initial pilot and feasibility study at the University of Pennsylvania DERC, two outstanding investigators in widely divergent fields of investigation began what has become an extremely fruitful collaboration on research directed toward subcellular studies of the islet cell.

The centers have also been instrumental in undertaking research on the psychosocial problems of the diabetic patient, a heretofore neglected area of research. The University of Virginia DRTC has two pilot and feasibility studies investigating methods for improving patient compliance. The Vanderbilt and Joslin DRTCs are also supporting work in this area.

Activities of the Training and Information Transfer Component at the DRTCs. Prior to establishment of the DRTCs, each of the center institutions provided training and educational experiences based primarily on traditional didactic methods. With the establishment of the DRTC program, centers have been able

to enlist the aid and expertise of specialists with knowledge and experience in the most contemporary methods of health education and evaluation. This has enabled centers to develop and test innovative and more effective ways of approaching training and education of health professionals concerned with the diabetic patient.

Although biomedical research is a primary focus at the DRTCs, the long range goals of these centers include the transfer of this knowledge base from the research laboratory to the wide spectrum of health professionals engaged in the care, education, and management of the diabetic patient. This includes development of a national resource of programs which provide excellent and effective training of health professionals on the most current methods of diagnosis, management, and treatment of diabetes and its complications. Developed and tested training and information transfer activities are then disseminated to a broader spectrum of health professionals. Thus, the product of this center component is development of "exportable" programs for training health professionals.

A recent example of a successful export of a program comes from the DRTC at Washington University St. Louis. A dietitian from Scott Air Force Base in Illinois who attended one of the basic courses offered by this DRTC subsequently invited a teaching team from the center to explore the possibility of the Base developing its own educational team and a syllabus relevant to its particular needs. Using the center guidelines for a systematic approach to patient education, the Air Force Base has now established a diabetes patient education program and a referral system through which patients can enter this program. The USAF has officially approved this diabetes education program and it will become part of the Air Force Dietetic Internship program for dietitians from Air Force Bases throughout the country.

In the course of the development of these activities, videotapes, slides, manuals, and new brochures have been produced. Additionally one center (University of Michigan) has compiled and published a catalog entitled "Audiovisual Resources for Diabetes Education", and has plans to update this resource annually.

Within the Training and Information Transfer Component at the Einstein-Montefiore DRTC, the formation, preparation, and implementation of a multidisciplinary Diabetes Education Team represents a significant accomplishment. This team provides teaching, evaluation, and consultation services to both its parent medical center and local agencies. Comprised of an internist, social worker, nutritionist, nurse, and podiatrist, the Diabetes Education Team has undertaken a variety of activities that make it the focal point of training and information transfer at this center. The team by giving equal importance to the psychosocial and biomedical aspects of diabetes serves as a "model" not only for the education of the health professional, but also for the diabetic patient.

The training and transfer components of DRTCs are also benefitting medical education in their host institutions. For example, the Vanderbilt DRTC offers fourth year medical students a clinical elective in diabetes which differs

substantially from the traditional curriculum. Students observe and work in the DRTC Model Demonstration Unit two half days a week for four weeks. Student time is divided between lectures, participation in the care of both adult and pediatric patients, and case discussions with the health care team. Medical students attend lectures given by different health professionals: a nurse, a dietitian, a clinical psychologist, and physicians. Nursing, dietary, psychology, and education students also attend the medical student lecture series, enhancing the team approach to the care of the diabetic patient.

The transfer of useful approaches to diabetic patient care is enhanced by developing relationships between the centers and other Federal agencies which support diabetes-related programs. DRTCs and nearby CDC Diabetes Control Projects have been exploring possible collaborative efforts and ways to exchange information. The centers also cooperate with the Health Services Administration's Indian Health Service (IHS) Diabetes Program. The IHS Diabetes Program is examining the feasibility of providing education and training on diabetes to Indian Health Service personnel at five selected locations. Arrangements among the Indian Health Service, the NIAMDD, and the DRTCs have been made which will allow the Director of this program to utilize some center personnel in conducting these training programs. This experience will be valuable to the DRTCs in evaluating the effectiveness of certain training programs in populations and cultures otherwise not available to the centers. This collaboration will also benefit the IHS program by providing needed medical expertise in diabetes for its training program.

Thus, the DRTCs offer a unique resource for interaction between traditional biomedical research and health care delivery activities and are clearly excellent places for the development of programs which reflect the latest research advances applied to training and continuing education for health professionals. However, the widespread application of these programs is clearly outside the mandate of the NIH. Therefore, funding for the application of such programs must come from the other Federal, State, and/or private agencies with interest or responsibilities in health care delivery. Realization of the full potential of the DRTCs in the future will depend on the full cooperation and support of other Federal agencies through their health care delivery programs and activities.

National Diabetes Information Clearinghouse

NIAMDD established the National Diabetes Information Clearinghouse (NDIC) in September 1978 to serve as the central resource for the collection and dissemination of information about educational and scientific materials, programs, and other resources relevant to diabetes. To this end, the Clearinghouse strives to increase the availability of information for health care professionals, for patients, and for their families; increase community awareness and understanding of diabetes as a major health problem; and establish a viable system for speedy communications about new developments, techniques, and programs in diabetes among all sections of the diabetes community.

Since its inception, the Clearinghouse has abstracted and indexed almost 1600 diabetes educational brochures, booklets, etc., for health care professionals,

people with diabetes, and the general public. The materials have been categorized into subject areas and compiled into 20 topical annotated bibliographies (5 in press). During the first half of FY80, the Clearinghouse distributed almost 6,000 of these publications. Three bibliographies -- Educational Materials For and About Young People with Diabetes, Cookbooks for Diabetics, and Teaching Guides for Diabetes Education Programs--easily outdistance all the other publicatons in number of requests. So far, five bibliographies have had to be reprinted while another is being updated.

These bibliographic listings not only provide information but also identify areas needing more educational materials. An example is the severe shortage of diabetes materials for adults with limited reading ability and for other minority populations. NDIC provided needed support to the Indian Health Service to develop and field test a diet manual for one such group, the American Indians living in the Southwestern United States.

NDIC continues to cooperate closely with other Federal and State agencies, voluntary organizations, and health professionals to develop strategies to increase community awareness and understanding of diabetes as a major health problem and to encourage effective patient, family, and community educational programs. For example, NDIC has supplied the Vermont ADA affiliate and the Maine State Department of Health Diabetes Project with enough selected bibliographies to be distributed to every hospital in these states.

In addition, in FY80 NDIC has coordinated four diabetes-related workshops sponsored by the Diabetes, Endocrinology, and Metabolic Diseases Cluster and is currently involved in providing logistical support for three more meetings and conferences. These meetings enhance communications about new developments, techniques, and programs in diabetes among the scientific community.

The NDIC is also compiling a glossary of diabetes terms and is editing the manuscript, Behavioral and Psychosocial Issues in Diabetes: Proceedings of the National Conference.

Publications:

Hamburg, B.A., Lipsett, L.F., Inoff, G.E. and Drash, A.L., (eds.) Behavioral and Psychosocial Issues in Diabetes: Summary of Proceedings of the National Conference. Diabetes Care 3: 379-381, 1980.

Hamburg, B.A., Lipsett, L.F., Inoff, G.E. and Drash, A.L., (eds.) Behavioral and Psychosocial Issues in Diabetes: Proceedings of a National Conference GPO # 80-1993 (in preparation).

Lipsett, L.F. Diabetes: An Overview. Behavioral Medicine Update. 2 (3), August 1980 (in press).

National Diabetes Data Group

The Data Group serves to foster, coordinate, and integrate national resources for the collection and analysis of data on diabetes in order to provide a sound basis for the development of scientific priorities and the planning of public health programs. The Data Group also continues to provide the federal focus to which both the lay and scientific communities turn for accurate, reliable statistics on diabetes.

The National Diabetes Data Group consists of a nationwide group of expert advisors including epidemiologists, individuals with expertise in the research, nutritional, and socieconomic aspects of diabetes, as well as representatives from federal agencies (the National Institutes of Health, the Center For Disease Control, the National Center For Health Statistics, and the National Diabetes Advisory Board) and the private sector (the American Diabetes Association and the Juvenile Diabetes Foundation).

A major problem in assessing the scope and impact of diabetes has been the lack of a satisfactory diagnostic and classification scheme for the various forms of the disease. To address this issue, the Data Group sponsored an international work group which formulated a consensus on a uniform classification of diabetes, appropriate diagnostic criteria, and standard terminology to be used as a guide for clinical and epidemiologic research. During the past year, these proposals were endorsed by the American Diabetes Association and were circulated among diabetes associations in other countries in order to achieve an international consensus. Recently, this effort has culminated in adoption of the recommendations of the Data Group by the Expert Committee on Diabetes of the World Health Organization.

In response to requests by the National Diabetes Advisory Board, the Data Group prepared an analysis of current research and knowledge on diabetes in black Americans. Although the rate of diabetes and the mortality from diabetes in this group is much higher than whites, it was found that virtually no research is being conducted to elucidate the reasons for these racial differences. The Data Group also assessed the changing mortality rates for diabetes, particularly the rapid decline in the cardiovascular disease death rate among diabetics. An analysis of multiple-cause-of death data from the State of Iowa, uncovered a shift away from the cardiovascular diseases and toward noncardiovascular conditions as causes of death of diabetics. In addition, there appears to be a redistribution within the cardiovascular causes such that ischemic heart disease and cerebrovascular disease are declining as causes of death while congestive heart failure is gaining more importance. In addition the Data Group developed a compilation of statistics on morbidity and mortality due to the complications of diabetes and on aspects of medical care and health services for diabetics. This document was used by participants in the Board's "National Conference on Health Services for Diabetics" as the quantitative basis for the recommendations on programs to reduce morbidity and mortality from diabetes and its complications.

The Data Group continued to work in close collaboration with the National Center for Health Statistics in assessing the national data on diabetes collected by that agency. With the Division of Mortality Statistics, the Data Group updated death rate figures for diabetes and documented the continuing decline in diabetes mortality in the U.S. With the Division of Health Examination Statistics, the Data Group is analyzing oral glucose tolerance tests on a statistical sample of the U.S. population to develop norms and standards for plasma glucose levels and to determine the prevalence of undiagnosed diabetes. This Division will also be conducting a study of Hispanic-Americans, and the Data Group is providing advice on the diabetes component of this survey. An analysis of the changing prevalence and incidence of diabetes over the last forty years is being conducted in collaboration with the Division of Health Interview Statistics.

A workshop on a newly-discovered phenomenon in diabetes, "Chlorpropamide - Alcohol Flushing", was organized by the Data Group and co-sponsored by the National Diabetes Information Clearinghouse. This phenomenon may serve as a genetic marker for one type of noninsulin-dependent diabetes, and, since it may be mediated by neuropeptides, has potentially important implications for a role of the central nervous system in the etiology of diabetes. A report of the workshop was prepared for distribution to the research community.

Publications:

National Diabetes Data Group: Classification and Diagnosis of Diabetes Mellitus and other categories of Glucose Intolerance. Diabetes 28: 1039-57, 1979.

National Diabetes Data Group: Selected Statistics on the Health and Medical Care of Diabetes. NIH Printing Office, June, 1980.

Harris, Maureen: Decline in The Cardiovascular Death Rate Among Diabetics (abstract). Proceedings of the 40th Annual Meeting of the American Diabetes Association. June, 1980.

Harris, Maureen, and Thomas Drury: The Changing Prevalence of Diabetes in the U.S. (abstract). Proceedings of the 40th Annual Meeting of the American Diabetes Association. June, 1980.

Harris, Maureen: Classification and Diagnosis of Diabetes Mellitus, in Diabetes Mellitus - Problems in Management, J. David Schnatz, editor. Addison-Wesley Pub. Co., Menlo Park, CA 1980.

Harris, Maureen: New Standards for the Classification and Diagnosis of Diabetes. Journal of The American Medical Association 243: 2296-7, 1980.

Endocrinology, Metabolic Diseases, and Resources Programs Branch

This Office coordinates the Endocrinology, Metabolic Diseases, Research Training and Development, and Resource Development Programs of the cluster. In addition, the office serves as the focus for planning and implementing special initiatives and for administering the NIAMDD cystic fibrosis-related activities.

Metabolic Diseases Research

The primary objective of this program is to support investigator initiated basic studies on the role of proteins and enzymes in both normal and pathometabolic processes. Thus, research on membrane structure, biological transport and enzymatic mechanisms, with a special emphasis on enzyme structure, function, and regulation constitute the largest thrust of the program. Given the almost universal involvement of proteins and enzymes in the normal as well as the diseased state, additional major areas of research support reach into the etiology and pathogenesis of various metabolic diseases. Highlights of ongoing research reported during FY80 are summarized below.

Functions of Proteins and Enzymes. Myosin, a large protein which forms thick muscle filaments, plays an important role in muscle contraction. The enzyme myosin adenosinetriphosphatase (ATPase) is involved in the muscle relaxation process. The structure of ATPase's active site and thus its mechanism of action is as yet unknown. One group of investigators succeeded in demonstrating that two sulfur containing thiol groups, one on each myosin head, are independent from the active site. Through structural manipulations, they demonstrated a mechanistic connection between these thiol groups and the active site of the enzyme. During chemical reactions involving the thiol groups, the active site "traps" an adenosinetriphosphate or analog molecule, enabling researchers to study the active site extensively. These findings will help elucidate the muscle contraction-relaxation mechanism.

Other research is focusing on the active site of acetylcholine, a general transmitter of nerve impulses at the motor end plates of skeletal muscle fibers. While studying the activity of choline acetyltransferase, the enzyme responsible for synthesis of acetylcholine, researchers found evidence suggesting that choline acetyltransferase contains two classes of reactive thiol groups, neither of which are required for enzyme activity. The researchers identified a chemical compound which forms tight complexes with the active site of the enzyme, while reacting with the neighboring thiol group. This finding will allow more thorough active site studies and will significantly accelerate progress toward understanding the mechanism of acetylcholine formation and of impulse transmission in skeletal muscles.

Glycogen, a high molecular weight branched polymer of glucose, provides the fuel needed for production of high-energy phosphate in tissues like muscles. In some cases, "supranormal" levels of skeletal muscle glycogen (a phenomenon called glycogen supercompensation) occur in man following exhaustive physical exercise. The increased glycogen stores are thought to result from increased glycogen production rather than diminished utilization. Researchers investigating the role of the enzyme glycogen synthase in supercompensation have found that glycogen synthase activation increases significantly in exercised muscles and remains somewhat elevated for approximately two days. Experimental results suggest that besides the totally dephosphorylated active form I and the totally phosphorylated inactive form D, there are intermediate, i.e. partially phosphorylated, forms of glycogen synthase in muscle and that supercompensation can be explained in part by formation of these intermediate forms. Although further studies are still needed, the investigators have demonstrated the usefulness of the exercise model in describing in vivo enzyme activation.

Spectroscopic Studies of Proteins, Enzymes, and Metal Ions. Many researchers have begun to use nuclear magnetic resonance (NMR) spectroscopy to study peptides, proteins, and enzymes in solution. By utilizing the differential behavior in a magnetic field of hydrogen, carbon-13, and fluorine-19, researchers have contributed significantly to the study of membrane transport and enzyme structure.

NMR has been used to study ion transport across cell membranes. In the human body, choline associates with phosphate and fatty acids as phospholipids or phosphatidylcholines, which are indispensable membrane components. Gramicidin A facilitates the diffusion of monovalent metal ions across such membranes by forming transmembrane channels situated between phospholipid molecules and made up of two molecules of gramicidin A connected head to head. Research has determined the conformation of this polypeptide antibiotic in phosphatidylcholine vesicles and established that they are connected through amine terminals. These findings are important in advancing our understanding of membrane organization and permeability in vitro.

Calcium has numerous roles in mediation and regulation of metabolic processes. It is a needed component of hormonal action and stimulation. By using a fluorescent probe, whose degree of fluorescence depends on the concentration of calcium ions in its vicinity, a group of investigators were able to localize the hormone-responsive pool of calcium ions in mitochondria of isolated liver cells. This important finding could contribute significantly to our knowledge of regulatory action of hormones like glucagon, vasopression, and norepinephrine which stimulate glucose formation from amino acids.

Methods for Separation, Identification, and Quantitation of Peptides, Proteins, and Enzymes. Preserving human enzyme activity in vitro for long periods is essential for successful biochemical research, especially when rare metabolic diseases are studied. One research group developed a method for solubilization and stabilization of human liver glycoprotein sialyltransferase, an enzyme of great importance in abnormal lipid metabolic processes relevant to Tay-Sachs disease.

Electrophoresis on polyacrylamide gels in the presence of a detergent is an extremely useful method for separation of peptides and proteins from plasma, cell extracts, urine, etc. One method for localization of spots belonging to the separated compounds is achieved by staining the gel with a dye called Coomassie blue. This stain, however, can interfere with the recovery and subsequent analysis of the separated peptides or proteins. A group of researchers developed a method which can recover unstained proteins with molecular weights up to 13,000 from the spot of choice. This is a significant advance in the separation and identification methodology of peptides and proteins.

Recent progress in culturing antibodies and using their propensity to attach to antigens enabled researchers to identify, localize, and quantify a large number of important peptides, proteins, and enzymes. One group utilized immunofluorescence (where the antibody is labeled with a fluorescent probe) to localize the enzyme adenosine deaminase (ADA). These researchers further

elucidated the role of ADA in muscle metabolism through showing that it is bound to the myosine section of the muscle fibers. Another group utilized a sensitive immunoassay to identify the distribution and comparability of ADA-binding proteins, thought to be responsible for the molecular heterogeneity of ADA from human tissue. Results indicate that binding proteins from liver, lung, spleen, fibroblasts, plasma, and urine react with the same antibody to kidney complexing protein, suggesting the possibility that most of these various binding proteins might be generated from the same gene. Forwarding our knowledge of human ADA has extraordinary importance given the major role ADA plays in severe combined immunodeficiency diseases.

Discovery of SAA, a small subunit of the fibrous protein amyloid, has broad ramifications for the diagnosis of metastatic cancer. Using radioimmunoassay, a group of researchers demonstrated that levels of SAA were elevated in sera of patients with solid tumors with distant metastases, in all tested patients with advanced lymphoma, and in those with myelocytic leukemia. The SAA level decreased in patients with disseminated neoplasia responding to treatment.

Inborn Metabolic Diseases. Heritable severe combined immunodeficiency disease appears to be due to adenosine deaminase (ADA) deficiency which results in the accumulation of toxic adenosine and deoxyadenosine. Adenosine appears to cause a suicide-like inactivation of an enzyme, S-adenosylhomocysteine hydrolase, resulting in the accumulation of S-adenosylhomocysteine causing secondary toxic effects. These findings add new dimensions to our understanding of this complex disease.

Cystinosis is a storage disease, resulting in cystaline deposits of the amino acid cysteine in eye, bone marrow, lymph gland, and rectal mucosa tissue. The disease can result in polyuria, rickets, acidosis, and hypokalemia which may lead to severe growth retardation, uremia, and death during the first decade of life. Measuring levels of cysteine in special cell organelle preparations from cystinotic and from normal cells, researchers have found that cystinotic preparations contained four to five times more cysteine than the normal preparations. These findings may lead to a better understanding of the role of cell organelles in cysteine metabolism.

Endocrinology Research

A major emphasis of the Endocrinology research program is to support studies directed at determining how hormones act at the cellular and subcellular level in the organism. This program supports a number of studies that attempt to determine the relationship of hormonal structure to function and the role played by cellular and subcellular membranes in hormone action. Other areas of emphasis include studies on secretion mechanisms of hormones, hormonal precursor molecules, and the synthetic processes used by the cell to produce hormones. In addition to these basic studies, the program also supports a number of clinically related studies on the pituitary, thyroid, parathyroid, and adrenal gland hormones. Studies on more recently discovered hormones, e.g., peptides from the hypothalamus such as endorphins and encephalins and on hormone-like substances, such as prostaglandins, are also supported.

Highlights from the work supported by the Endocrinology Research Program follow.

Studies Using Recombinant DNA Techniques. In the report for 1979 we noted that rat growth hormone (GH) has been produced in bacteria using recombinant DNA techniques. We can now report that human growth hormone has also been produced by the same methods. Although some problems remain in using this method for large scale production of growth hormone, this first step is an important one in the effort to alleviate the shortage of the hormone which is so critical to children with short stature caused by growth hormone deficiency.

The recombinant technique is valuable not only for hormone production, but also as a tool useful in basic studies. For example, recombinant techniques have been used to determine gene structure and how genetic information is transcribed and translated into protein production. Previous studies by NIAMDD-supported researchers had shown that thyroid and glucocorticoid hormones can increase the concentration of growth hormone messenger RNA. Current research on isolated cell nuclei indicates that these hormones also increase the number of RNA polymerase molecules engaged in gene transcription. Since the enzyme RNA polymerase catalyzes formation of messenger RNA, the evidence mounts that both thyroid and glucocorticoid hormones are important in gene transcription.

However, other research shows that thyroid hormone acts on the mitochondria of liver cells. By injecting triiodothyronine into hypothyroid rats, it was shown that mitochondrial oxidative phosphorylation increased. These results, together with those above, support the contention that the thyroid hormone may act on more than one part of the cell.

Brain Control of Endocrine Function. In recent years it has become apparent that the hypothalamus produces various releasing and inhibiting factors but little is known about how the rest of the brain relates to the hypothalamus with regard to neuroendocrine control mechanisms. Several studies are underway which may help our understanding of this process.

In one study the suprachiasmatic nuclei (SCN) were destroyed, following which blood samples were measured for plasma corticosterone levels. The data suggest that the SCN are essential for the circadian rhythms in pituitary-adrenal function and body temperature and that separate pacemakers may be present in these nuclei for these two periodic functions. The SCN may also control rhythmic TSH secretion from the pituitary.

Somatostatin inhibits growth hormone and TSH secretion from the anterior pituitary. In an effort to localize the area of the hypothalamus where somatostatin is produced, lesions were made in the medial preoptic-anterior hypothalamus area. Although elevated levels of growth hormone indicated that these lesions caused some depletion of somatostatin, their value suggests that other brain areas are involved in somatostatin production, and more localization studies are needed to complete the picture. These studies also show that TSH elevation accompanied GH elevation following lesion. This is further evidence that somatostatin affects TSH secretion and is one of the first pieces of evidence that TSH can be elevated by neural lesions.

Studies of TRF In Lower Vertebrates and Invertebrates. The hypothalamic hormone controlling TSH secretion, thyrotropin releasing factor (TRF) is a tripeptide amide, pryoglutaryl-histidyl-proline, discovered in 1969. This material appears to have physiologic roles in cold blooded vertebrates and in some invertebrate animals. In order to determine some of these physiological effects, one investigator has studied the role of TRF in snails. The hormone appears to have an osmoregulatory function by causing a pronounced water loss. It also reduces the uptake of sodium by the snail from the surrounding water. Studies are ongoing to see if the influence of this hormone affects the amounts of sodium pumped out of the animal.

It has been postulated that TRF may be a hibernating hormone in frogs because of the observation that TRF reduced salt transport in incubated skins from spring and summer frogs, but had no effect on skins from hibernating frogs. Furthermore the TRF content of ventral frog skin is highest in the fall just prior to hibernation, and lowest in the spring at the end of hibernation.

These two studies, while of interest, do not relate directly to mammalian or human physiology. They are important, however, because they indicate possible mechanisms of action of hormones which might go unnoticed ordinarily, and may eventually contribute to our understainding of the mechanisms involved in human hormone action.

Pituitary Hormone Studies. One grantee has discovered that newly synthesized prolactin is released sooner than hormone which has been manufactured and stored as cell granules. Using cell culture techniques and various methods of labeling the products, this investigator has demonstrated preferential release of newly synthesized prolactin under the condition studied due to preferential release of newly synthesized granules. The results can be explained by heterogenity in the mammotroph cell population: some cells are synthesizing and secreting the hormone at a much more rapid rate than others.

Another series of studies has shown that ACTH and endorphin are produced as part of the same common precursor molecule from the pituitaries of rats and mice. It can be shown that the entire molecule is made up of three smaller peptides, the B lipotropin, ACTH, and a glycoprotein fragment of unknown function. The B lipotropin molecule splits into B endorphin and A MSH. Thus one pituitary cell type produces several hormones from one larger precursor molecule, which is later split into smaller active fragments.

Hormone Production and Distribution Program. The institute continues to supply human Growth Hormone, human FSH, LH, TSH prolactin to qualified researchers for clinical research. In addition, pituitary hormones from rat and sheep, and to a lesser extend beef and pork are available for basic research. Radioimmunoassay kits for these hormones are also distributed to the research community.

Cystic Fibrosis (CF)

During FY80, efforts were continued to increase investigator awareness of the needs and opportunities in CF research. The Director, NIH, distributed information on CF research to several thousand investigators and invited them

to obtain additional written materials from this office. Such materials included a series of three booklets entitled "Cystic Fibrosis: A Disease in Search of Ideas", which summarizes current knowledge and research needs in the areas of basic science, clinical science, and model systems related to cystic fibrosis. Although the full impact of this publicity cannot be gauged until next year, FY80 saw more than a 20 percent increase over FY79 in CF-related research applications assigned to the institute, representing a 240 percent increase over FY78.

Previous annual reports have summarized efforts to develop ways of detecting asymptomatic carriers of the CF gene (heterozygotes) and of performing prenatal diagnosis of the disease. A report several years ago that fibroblasts from CF patients are less easily killed by dexamethasome than are fibroblasts from normal individuals has drawn considerable interest and a number of investigators are continuing to explore this phenomenon. Similarly, work has continued to explore the abnormalities previously reported in the α-2-macroglobulin of CF patients. Very recently, α-2-macroglobulin from CF patients, heterozygotes, and normal controls has been purified and a number of comparative tests performed. Differences were found in carbohydrate composition and in the degree to which the α-2-macroglobulin binds to concanavalin A and wheat-germ agglutinin. Work has also continued to develop a test for CF using amniotic fluid obtained by amniocentesis. This test is based on the observation that protease activity is lower in the amniotic fluid of CF pregnancies than in normal controls. Over 1,300 pregnancies have been screened for protease activity, and those at risk for CF are being monitored. Results to date are extremely encouraging.

A number of basic research projects are examining the secretory process in normals and in CF patients. The structure and function of the sweat gland is a major focus of interest in these studies because of the high concentration of electrolytes found in the sweat of CF patients. The search continues for a postulated single basic defect in CF patients, thought by many investigators to involve the transport of ions and fluids across membranes.

Training and Career Development Program

The Training and Career Development Program continues to pursue its mission of assuring that an adequate supply of well-trained scientists becomes available in the areas of diabetes, endocrinology, and metabolic diseases, with a particular focus on the development of physician-scientists to carry on clinical research.

Career Development Awards. In 1978 a Special Emphasis Research Career Award (SERCA) was initiated jointly with the National Heart, Lung, and Blood Institute to encourage young physicians interested in research to develop research skills in cardiovascular, metabolic, and endocrinologic aspects of diabetes. The award includes five years of salary support and a limited allowance for research expenses. Nine of these awards were funded in 1978 and 1979. In 1980, parallel awards were initiated in two additional interdisciplinary areas: one in obstetrical, neonatal, and pediatric aspects of diabetes, funded jointly with the National Institute of

Child Health and Human Development, and the other in the area of diabetes in the elderly, funded jointly with the National Institute on Aging.

In addition to the SERCA awards, the cluster made a number of Clinical Investigator Awards (CIA). This award, initiated in the cluster in 1977, provides three years of salary support plus a research allowance to individuals holding clinical degrees. The allowance was recently raised from a maximum of $10,000 to a maximum of $20,000 per year.

Research Training. Training is supported under the authority of The National Research Service Act through the individual award for postdoctoral training and the institutional training grant for postdoctoral training and limited pre-Ph.D. training. FY80 saw the introduction of a new type of institutional training award focused exclusively on short-term (usually 3-month) research experiences for students currently enrolled in clincial degree programs. Also initiated in the past year, the Senior Fellowship supports mid-career scientists to gain additional training to augment their research skills. Finally, the cluster, together with the Fogarty International Center, initiated a Special International Fellowship in Diabetes to allow mid-career scientists in diabetes-related areas to study and work in laboratories abroad. Four awards have been made to date.

In January, 1980, the New Investigator Research Award, previously restricted to the area of diabetes only, was expanded to span all research areas of the cluster. These awards are now administered in the relevant research grant programs rather than in the Training and Career Development Program.

Research Resources

The cluster continues to support the development and distribution to qualified investigators of various research resources not available from commercial sources. Limited quantities of gastric inhibitory polypeptide, chicken insulin, guinea pig insulin, and desoctapeptide are now available, and the program is considering ways to meet other resource needs as funds allow. Animal models continue to be an important focus of interest. Although the Institute continues to plan for expanded activities in the development of models, budgetary limitations prevent the growth of this program.

ARTHRITIS, MUSCULOSKELETAL AND SKIN DISEASES PROGRAM
ANNUAL REPORT - 1980

ARTHRITIS RESEARCH AND TRAINING PROGRAMS

The individual investigator initiated research grants in the Arthritis Program can be categorized by four major areas of emphasis. The first includes general approaches to the rheumatic diseases concerned with etiology, metabolism, inflammation, humoral and cellular disturbances, autoimmunity and specific antibodies. The second is research differentiated to categorical diseases such as rheumatoid arthritis, systemic lupus erythematosus, degenerative joint disease, heritable disorders of connective tissue, etc. The third is relatively undifferentiated research related to articular cartilage, collagen, mucopolysaccharides, basement membrane, and synovial tissue fluids. Fourth, and lastly, studies associated with the structure, function, and metabolism of skeletal muscle, together with the physical and biochemical events associated with excitation and contraction.

Highlights and summaries of research objectives and results from individual research projects along with current proposed researches are reported within areas of major interest that comprise the program.

Etiology of Arthritis. Lyme arthritis continues to command a great deal of attention in the search for an infectious etiology for arthritis. This type of arthritis appears to be a tick-transmitted, immune mediated epidemic disorder in children and adults which usually begins in the summer with a characteristic skin lesion that may be followed by a syndrome that includes a migratory, intermittent, or chronic arthritis. The investigative team at Yale initially recognized the disease and defined many of its clinical aspects and implicated a vector, Ixodes dammini. During the current grant period there will be efforts to control the illness and ascertain prognostic factors, treatment regimens, and the long-term effects of the disease. In addition, further identification of patients through active surveillance and the specification of attack rates within sample human and animal populations will be undertaken. An animal model for Lyme disease will be sought as well as exploration of animal hosts and the natural environment of the implicated vector. Finally, the possible roles and inter-relationships between circulating immune complexes, lymphocyte dysfunction, blood coagulation abnormalities and immunogenetic determinants in the pathogenesis of the disease will be explored.

A current project in which rheumatoid synovial cells have been cocultivated with human embryo lung cells or fetal rabbit synovial cells has for the first time consistently demonstrated viral particles by electron microscopy in these cocultivations. Inoculation of cocultivation extracts into the brain of suckling mice produces severe pathology and death in the mice with recovery of virus titers. The immediate objectives are to confirm the presence of the virus in the rheumatoid synovial cells, to obtain sufficient virus titers for characterization of the agent, and to demonstrate its relevance to the etiology of rheumatoid arthritis. An animal model will be attempted by injection of viral material into rabbit joints followed by various virologic,

histologic, and biochemical studies.

Another thrust to identify microbial agents suspected to be etiological agents in rheumatoid arthritis and systemic lupus erythematosus proposes to identify and characterize microbial DNA in plasma and tissues of such patients with techniques based on nucleic acid hybridization, DNA-DNA renaturation, nucleic acid probes, and others. Two approaches are now being used. In one, DNA will be isolated from the circulation to determine if it is of foreign origin. Earlier studies isolating DNA from a number of subjects with SLE did not result in its renaturing with known DNA, suggesting that a base sequence difference may be present. Further studies will be conducted to see if the explanation for this is in the methods used or if there are true differences between based sequences and those of human nuclear DNA. The second approach will be to prepare DNA probes of high specific activity to attempt to detect DNA from microorganisms in tissue.

Rheumatoid Arthritis. Support is being provided for work in three major areas related to autoimmunity in rheumatoid arthritis. The ongoing work in these areas seeks to: characterize a rheumatoid factor plaque-forming cell system to determine whether T-cell immunity to IgG exists in the peripheral blood lymphocytes of patients with rheumatoid arthritis; to characterize the RA-associated nuclear antigen generated by EB virus in lymphocytes; and to assess the relative significance of rheumatoid factors, other immune complexes, the rheumatoid arthritis nuclear antigen, anti-lymphocyte antibodies, and anti-EB antibodies on the course of rheumatoid arthritis. The work in progress will very likely elucidate the possible role of T-cells and macrophages in the development of cells which synthesize rheumatoid factor. Proposed analyses of the quality of immune complexes in rheumatoid arthritis are among the few systematic studies of this problem now available and the type of work in this proposal could have a high probability in identifying new antigens which could provide an important breakthrough in this field.

Systemic Lupus Erythematosus. Anti-lymphocyte antibodies (ALA) have been found in patients with systemic lupus erythematosus (SLE) and in a significant percentage of both their blood and non-blood relatives. The project is being supported to gain further information on the relationship between ALA and disease activity in SLE families, the mechanisms of their production and their effect on monocyte and lyphocyte function. A longitudinal study of SLE families will determine the correlation between disease activity and subtypes of ALA with specificity for major histocompatibility antigenic structures, $beta_2$ microglubulin, and antigens expressed on brain cells and on proliferating lymphocytes. Antibodies with these specificities will be isolated and examined for their effect on the ability of monocytes and T and B lymphocytes to perform in in vitro tests. The effects of ALA will be compared to those of xeno-antibodies with similar specificities. Also studied further will be the role of prostaglandin E_2 (PGE_2) in feedback control of monocyte and lymphocyte function. Emphasis will be placed on the mechanism behind the excessive in vitro PGE_2 production by monocytes found in patients with Hodgkins disease, SLE, and rheumatoid arthritis. Increased incidence of lymphocytotoxic antibodies (LCA) in SLE relatives has been confirmed and also found in laboratory technicians exposed to SLE sera and in relatives of patients with Hodgkins disease. A correlation has been found between LCA and anti-nucleic acid

antibodies in both SLE probands and their relatives. LCA and anti-nucleic acid antibodies were found in the IgM class in relatives but in IgM and IgG classes in probands, and both SLE probands and relatives had ALA with specificity for the major histocompatibility antigens.

Regulation of anti-nucleic acid antibodies in NZB/W mice is the central theme of a project which is focussed on the roles of sex hormones and the thymus in antibody production and transition from IgM and IgG antibodies. Androgens and estrogens have been demonstrated to have significant and opposite effects on the course of the autoimmune disease in NZB/W mice. Castration and hormone treatment studies in these mice have shown that androgens suppress and estrogens enhance both the disease and the production of anti-nucleic acid antibodies. Additional findings suggest that male thymus, but not female thymus, contains cells that suppress these parameters. There is documentation for an orderly predictable progression of the production of anti-IgM and anti-IgG antibodies in these mice and similar correlation has been shown between disease and immunoglobulin autoantibodies in patients with SLE. Progess here has been very good. The connection between the IgM and IgG switch and the development of autoimmune disease in these animals is very interesting. The influence of sex hormones is also of great interest, since it offers an explanation for the female preponderance in SLE.

Osteoarthritis. The enzymatic mechanisms by which the extracellular matrix of human and animal cartilage is degraded is the main objective of a research group seeking to characterize and quantitate several proteolytic enzymes in normal and diseased cartilage. The work has focused on the identification and purification of various proteases in human articular cartilage and bovine nasal cartilage. A neutral metalloprotease activity was found in the culture medium from rabbit articular cartilage chondrocytes. The protease attacked a proteoglycan subunit prepared from bovine nasal cartilage. The viscosity and sedimentation coefficient of the substrate were reduced by the enzyme. The enzyme can be inhibited by EDTA and ophyloantheroline and stimulated by cobalt and calcium and in many respects resembles a neutral metalloprotease that has been previously described in human articular cartilage.

It was also shown that the neutral proteinase of rabbit chondrocytes appears in the culture medium in a latent form. This latent enzyme is quite similar to other latent enzymes in that latency is due to enzyme-inhibitor complexing, which can be reversed by several means. Adding culture medium to activated enzyme results in inhibition, indicating an excess of inhibitor over enzyme. It was further shown that these chondrocyte cultures produce collagenase which can degrade both type I and II collagen. However, cells that are most chondrocyte-like in terms of producing only type II collagen are least active in secreting collagenase. This would suggest that the production of collagenase in osteoarthritis may involve a change in the metabolism of chondrocytes.

Another study will continue to examine collagen synthesis in osteoarthritic cartilage. It is directed to the study of molecular mechanisms by which somatomedins regulate chondrocyte growth and matrix formation. The study is based on earlier observations that a slow but substantial increase

in the responsiveness of chondrocytes to serum stimulatory factors occurs over a period of weeks during in vitro culture. These studies of the effects of somatomedins were initiated with the observation, that as rabbit articular slices are maintained in culture in the presence of somatomedins, there is a gradual, impressive increase in the chondrocyte response to exogenous somatomedins. This raises the possibility of positive feedback control and induction of somatomedin receptor activity. The relationship between these observed influences of somatomedin and the etiology of osteoarthritis remains obscure at this juncture.

Immune Disturbances in Rheumatic Disease. Current projects are exploring certain key areas which may contribute to a better understanding of immune mechanisms in a number of connective tissue diseases. The attempt to define lymphocyte antigens unique for systemic lupus erythematosus may provide a valuable marker for disease activity among patients with this disorder.

If leukocyte antigens detected by anti-SLE reagents are in some way related to either differentiation antigens on leukocytes or a primary extrinsic etiology of the disease, then considerable application of such reagents towards the definition of a causative agent or tissue distribution of such materials would become available. Work on idiotypic antigens specific for rheumatoid factor could provide an additional serologic parameter to measure subclasses or subsets of antigen binding specificity within a large number of conditions associated with the generation of rheumatoid factor. The use of anti-idiotypes against rheumatoid factors could conceivably be a useful therapeutic modality in the treatment of patients with severe rheumatoid disease, in being able to shut off the initial immune reaction which may play an important role in the disease process itself.

In another series of studies based on indications that humoral and cellular immune mechanisms maintain the chronic joint inflammation in rheumatoid arthritis, perhaps in genetically susceptible individuals, the ultimate objective is to identify the antigen or antigens of immune complexes in patients with this disease. The events that initiate the disease may differ from the events that maintain the chronic disease process. Since immune complexes are frequently present in this disorder, an approach to a better understanding of rheumatoid arthritis may be the identification of the antigen or antigens that are involved in maintaining the disease process. Detailed evaluation by affinity chromatography for isolation of IgG rheumatoid factor was conducted. Nonimmunospecific protein-protein interactions between IgG in solution and IgG bound to an agarose matrix were evaluated and conditions were identified to minimize these unwanted interactions. Affinity chromatography under appropriate conditions was shown to be a reliable method for isolation of IgG-rheumatoid factor. Synovial fluid from patients with rheumatoid arthritis was shown to contain self-associating IgG-rheumatoid factor and additional studies are underway. A study of antigenic determinants involved in the self-association of IgG-rheumatoid factor showed that certain fragments (Fc) of these molecules do not possess unique antigenic determinants that would favor self-association. Therefore, the self-association of IgG rheumatoid factor would appear to be on the thermodynamics of the two antigen-antibody bonds formed in this interaction. The self-associated IgG-rheumatoid factor molecules have the capacity to activate complement, but relatively

ineffectively. The IgG-rheumatoid factor complex can also block complement-fixation by other immune complexes containing IgG by binding to the Fc fragments of these molecules.

Collagen and Connective Tissue. An important program is being continued to elucidate ways by which the cross-links of collagen participate in its biologic role of imparting strength, form and support to the tissues. In addition, the investigators will examine the biologic diversity of collagenous proteins by characterizing unusual collagens from animals such as polychaete worms and marine mussels. These studies have provided detailed information about the structure of an unusual collagen derived from the cuticle of an annelid, and have served as a basis for evolutionary and genetic considerations in the phylogenetic development of collagens and procollagens. In addition, the delineation of the properties of a new vibrial collagenase and comparison of such properties with a well-known clostridial collagenase have provided insight into the conformational structure of collagen. The vibrial collagenase is considerably more efficient than the clostridial collagenase, and may be the more useful compound in commonly employed assays which measure collagen content in tissues. Studies with byssus fibers from a marine mussel may eventually provide a novel means for joining connective tissue structures such as fractured bones. The adhesive properties of the natural byssus material potentially allows it to be used as a surgical and dental restorative material. In other studies, the high specific activity purification of the enyzme lysyl oxidase under non-denaturing conditions is vital to studying its properties. Lysyl oxidase is essential for collagen and elastin crosslinking and , as such, is a major factor in determining the properties of these connective tissue proteins.

Muscle Structure and Function. Studies concerned with muscle contraction include projects concerned with contractile proteins, the contractile mechanism, the biology and regulation of contraction, and the mechanical control and energetics of contraction. Projects associated with the structure and function of muscle include myofibril structure, sarcoplasmic reticulum, regulatory proteins, and the structural basis and dynamics of cellular function and movement.

SKIN DISEASES RESEARCH AND TRAINING PROGRAMS

The Skin Diseases Program of the NIAMDD is responsible for the support and administration of research within the disciplines of dermatology and cutaneous biology. Several areas of emphasis include:

1. Metabolic studies of skin including effects of hormones and interaction with enzymes

2. Comparative structure and function of skin in man and other primates

3. Immunologically mediated cutaneous disorders, including atopic dermatitis, eczema, contact dermatitis, and vasculitis

4. Photobiology, photo-allergy, and phototoxic reactions

5. Vitiligo and other disorders of pigmentation

6. Psoriasis and kinetics of keratinizing tissues

7. Bullous diseases of the skin, including pemphigus, pemphigoid, dermatitis herpetiformis, and epidermolysis bullosa

8. Acne

9. Cutaneous manifestations of connective tissue disorders such as lupus erythematosus, scleroderma, and pseudoxanthoma elasticum.

The Skin Diseases Program encourages and supports research training through National Research Service Awards, both individual fellowships and institutional awards; Clinical Investigator Awards; and Research Career Development Awards.

Epidermolysis Bullosa

Major research thrusts within the program has for several years been concerned with psoriasis and other keratinizing disorders. Although a better understanding of the process of keratinization is critical to progress in such disorders of the epidermis, there appears to be increasing research interest in the importance of supporting structures of the skin, namely the connective tissue. An interesting overlap between the study of connective tissue and bullous diseases of the skin has become more evident recently with increased attention to the disease epidermolysis bullosa. Institute staff is currently planning with investigators in the field for a state-of-the-art workshop on epidermolysis bullosa and other blistering diseases of the skin. Such a workshop seems timely; it has recently been reported that increased human skin collagenase may be implicated in the pathogenesis of blister formation in recessive dystrophic epidermolysis bullosa. If a defect in the regulation of synthesis of collagenase were basic to the etiology of this disease, it might be expected that increased levels of this enzyme would be seen in fibroblast cultures. Indeed, grantees reported this year that fibroblast cultures from patients with recessive dystrophic epidermolysis

bullosa have an increased capacity to synthesize and secrete collagenase. In fiscal year 1980 program staff initiated interaction and cooperation between scientists studying blistering diseases of the skin and officers of the newly formed Dystrophic Epidermolysis Bullosa Research Association (D.E.B.R.A.); staff will cooperate with this new voluntary health agency in providing information on scientists and resources available for the treatment of this mutilating disorder.

Vitiligo

In April 1979 the NIAMDD announced the award of a four-year grant for the purpose of expanded vitiligo research. The grant, totalling approximately $2 million, was awarded to a consortium comprised of Yale University, Howard University, Bryn Mawr College, the University of Pennsylvania, the University of Massachusetts, and the Massachusetts Eye and Ear Infirmary. In its first progress report, investigators at Yale University reported that its pigment cell clinic is in operation and is attended by over 350 patients with vitiligo. More than 250 questionnaires from patients with vitiligo have been completed, and those relating to the psychological impact of vitiligo have been returned to the investigators at Bryn Mawr and the University of Pennsylvania, where the data are being entered on computers. Many of the patients with vitiligo have had special eye examinations; 75 of them have had hearing tests and blood drawn for tests for auto-antibodies and antipigment cell antibodies. Lymphocytes have been obtained to test the reactivity against allogeneic melanoma cells. Discovery of antipigment cell antibodies in two patients with vitiligo and mucocutaneous candidiasis has suggested that immune mechanisms are involved in the destruction of pigment cells. Investigators at Yale have extended these serologic studies to patients with vitiligo, melanomas, melanomas with vitiligo, and patients with mucocutaneous candidiasis without vitiligo. Three of seven patients with mucocutaneous candidiasis without vitilito had strongly positive antipigment cell factors in their serum. Lymphocytes from 49 patients with vitiligo, halo nevi, Vogt-Koyanagi-Harada Syndrome, or melanomas were tested in vitro for reactivity against allogeneic melanoma cells. Lymphocytes from patients with halo nevi or melanomas were strongly reactive in this assay. Lymphocytes from patients with vitiligo only were nonreactive. Either the circulating lymphocytes of patients with vitiligo are not sensitized to antigens on the pigment cell or the improper assay method is being used to detect sensitization. The data from over 150 patients reveal that 30% of patients with vitiligo have pigmentary abnormalities in their eyes. In addition, investigators have completed a study which indicates that a minimum of 7% of patients with uveitis have vitiligo, an important observation. Contrary to beliefs of the past, it is now becoming apparent that the destruction of pigment cells in the skin, nevi, eyes, ears, or leptomeninges at times may be sufficient to cause symptoms of pruritus, photophobia, dysacousia or meningimus.

In studies emanating from the Vitiligo Clinic and Melanoma-Oncology Clinic, which are conducted together, 15 of 100 patients with melanomas of Clark level III-V have been noted to have characteristic but bizarre arrays of nevi. These nevi are different from those described in patients with the BK Mole Syndrome. These patients do not report family histories of nevi or melanomas. One patient was found to have a melanoma in situ arising in one

of these unusual nevi. These data suggest that patients with certain types of nevi are at very high risk for developing melanomas, and such patients should be watched more carefully.

Photobiology

Dermatologists and other medical investigators are becoming increasingly concerned about the cumulative effects of both short- and long-wave untraviolet light on the skin. Increasing leisure time and sun exposure is causing damage as a result of ultraviolet radiation ranging from the redness and tenderness of acute sunburn to the wrinkling seen after years of sun exposure. Cumulative repeated exposure also can lead to skin cancer, the most common type of cancer in humans. In spite of the recent strides that have been made in studying the skin changes caused by sun exposure, additional detailed studies are needed to learn how sunburn is initiated at the molecular level and about the progressive degenerative changes that ensue.

An example of expanding photobiology research is a recently awarded grant to study the optics and phototoxicity of skin. Studies suggest that long-wave ultraviolet potentiates the biologic effects of short-wave ultraviolet and is believed to play a role in sunburn. Many sun screens do not significantly alter the amount of long-wave ultraviolet reaching the skin and the same wave length is transmitted through most window glass and many plastics while short-wave ultraviolet is not. Both toxic and allergic photosensitivity reactions appear to be mediated by long-wave ultraviolet. Experimental and epidemiologic evidence suggests that solar long-wave ultraviolet is a possible etiologic agent for certain kinds of cataracts in humans. The industrial hazard of long-wave ultraviolet exposure is increasing as long-wave ultraviolet-induced photopolymerization and photochemical reactions are used more and more in manufacturing processes. However, long-wave ultraviolet may also be beneficial when used in conjunction with photosensitizing drugs. New therapeutic possibilities using UVA for the treatment of chronic skin disorders such as psoriasis, mycosis fungoides, and eczema are under study. The treatment, designated as PUVA, consists of the oral administration of psoralen (photosensitizing chemical) followed by exposure to high intensity long-wave ultraviolet light (UVA). The efficacy of this treatment for severe psoriasis was first reported by Institute-supported grantees in 1974. By 1978 an estimated 35,000 Americans were receiving this treatment. Recognizing the need for a carefully controlled follow-up of patients treated with PUVA, the NIAMDD sponsored a collaborative study three years ago centered at the Massachusetts General Hospital and involving 16 university centers for a five-year follow-up of the patients treated with PUVA. In their progress report for 1978 there was no indication that any of the patients had demonstrated any significant toxic reaction or gross pathological changes. By early 1979, however, growing optimism about the efficacy of PUVA therapy had been tempered by an interim report of the long-term effects and safety of PUVA therapy in follow-up evaluation of 1,373 patients who had participated in the earlier short-term PUVA trials. The investigation confirmed that PUVA therapy was effective, clean and acceptable to patients. However, a concern expressed several years before seemed to be realized, namely, the possibility that long-term irradiation to skin could result in malignant

transformation. Among the 1,373 patients, the investigators found 48 epidermal cancers in 30 patients. This incidence was 2.63 times that expected in a matched control population. Patients previously exposed to ionizing radiation or who previously had had skin cancers were at even higher risk. Moreover, in lighter-complexioned patients cancers developed more readily than in those who tanned well. Because PUVA treatment had been successful and is highly acceptable to patients, and in view of the safety concerns and the possibility of additional undetected long-term side effects, the NIAMDD is encouraging grantees to continue careful selection of patients and monitoring of this still promising treatment.

Eczematous and Immunologic Diseases

In cooperation with the NIAID, greater attention is being directed to eczematous and immunologic diseases. It has become clear that the skin is a target for diverse assaults by the immune system. For example, patients with atopic dermatitis have a constellation of immunological defects: elevated serum IgE levels, depressed cell mediated immunity, and reduced or abnormal leukotaxis. Institute-sponsored grantees are currently trying to determine whether these immunological abnormalities represent the primary defect or only a secondary response to inflammation. They are also attempting to demonstrate why many patients with atopic dermatitis display cutaneous anergy. Why their skin fails to show normal antigenic responses is unknown. This deficiency may or may not be related to local skin factors or possibly to central immunologic defects. It will be important to determine whether the increased IgE production is regulated by specific T cell sub-populations or by other controlling mechanisms.

Physiological Studies

Institute grantees are attempting to correlate physiological and ultrastructural studies of cutaneous microcirculation with special emphasis on identifying structural parameters of sphincter regions in the microvasculature. This is part of an interdisciplinary basic research program in dermatology; it also includes the development of new non-invasive methods to test the strength of healing surgical wounds with time, an analysis of biochemical and functional properties of the basic protein of stratum corneum with special reference to how this protein relates to cellular morphology and cutaneous disease in instances where keratohyaline is absent. The physiology, pharmacology, and pathophysiology of the equine sweat gland unit in man is being investigated to determine:

1. the locus and mechanism of secretory adaptation with heat acclimatization;

2. differences in cholinergic and adrenergic responsiveness in normal, allergic, and atopic individuals; and

3. the applicability of iontophoresis as a method of delivery of drugs intradermally.

Quantitative pharmacokinetics of percutaneous absorption in human skin will also be studied.

Acne

In FY 79 Institute grantees announced a major breakthrough in the treatment of the most severe forms of acne. The dramatic response of severe and treatment-resistant cases of cystic and conglobate acne occurred following the use of oral 13-cis-retinoic acid. The mechanism proposed for the action of this drug is its marked inhibition of the synthesis of sebum, the secretory product of the sebaceous gland which is an indispensable link in the pathogenetic sequence leading to inflammation in acne. During the past fiscal year grantees have continued to investigate the mechanism involved in the action of 13-cis-retinoic acid on sebaceous gland function and will meet in the late spring of 1981 under the auspices of an NIAMDD conference grant to review the action of the oral retinoids on acne and disorders of keratinization.

MUSCULOSKELETAL DISEASES RESEARCH AND TRAINING PROGRAMS

The Musculoskeletal Diseases Program provides research support for basic and clinical studies of the musculoskeletal system in normal and diseased states. The program utilizes many funding mechanisms including regular research grants, career development awards, training grants, and contracts. The budget for these programs has increased to approximately $14.7 million in FY 80. These funds support approximately 185 new, competing renewal and non-competing awards. Inflation and a 10 per cent increase in applications received have resulted in funding of a limited number of the most meritorious grants.

The total number of training and manpower development programs has remained essentially constant during the past year. Career development awards have increased slightly while the number of individual fellowships has decreased. Clinical Investigator Awards remain level at three; however, several young physicians are contemplating applying next year. Institutional training grants have increased by one. The training profile continues to be a positive indicator for developing future researchers.

Conference support was increased in FY 80. In all instances the Musculoskeletal Diseases Program co-sponsored these conferences or workshops with other components of NIH, other federal agencies, and/or private organizations. These small expenditures not only provide stimulating interchanges among leading scientists, but also generate new research applications in promising areas. Two conferences dealt with basic bone metabolism and structure in the areas of calcium binding proteins and bone histomorphometry. Another workshop covered the latest microsurgical advances in bone grafts and reconstruction. In a continuing effort to encourage osteoporosis research the Steenbach Conference on Osteoporosis was sponsored in June 1980. New scientific understanding of this disease process gives hope for developing successful therapies and methods of prevention.

A major workshop was held on the topic of heritable connective tissue disorders with special emphasis on: osteogenesis imperfecta, Ehlers-Danlos Syndrome, and Marfan's Syndrome. Molecular genetics, basic biochemical defects, and clinical management were discussed. There was a call for a unified biochemical and clinical classification of these groups of patients in order to obtain sufficient sample sizes to analyze more clearly these complex syndromes. The Musculoskeletal Diseases Program will encourage investigators to submit grant proposals in this area.

Two conferences were held on biomaterials and joint implants. The First World Biomaterials Congress had presentations on several topics relevant to orthopedics. New areas of interest included carbon fibre implants, improved ceramics, cementless fixation, and a calcium phosphate bone growth framework. A workshop on implant retrieval and analysis described current research areas and failure mechanisms. In that over 150,000 artificial joints are implanted annually and in view of the need for improved durability, it is imperative to learn from the successes and failures of past implantations.

Bone Diseases

The bone disease component of the program consists of basic studies related to the growth, structure, and metabolism of bone and projects concerning specific disease states in bone. There is a strong interrelationship between the basic and more clinically oriented projects. Selected research projects in areas of acquired and inherited bone disorders are described in the paragraph below.

Because of the clinical prevalence and serious disability caused by osteoporosis, this metabolic bone disorder has been a major component of the program. An investigator at Creighton University is in the midst of a 20-year longitudinal study of a pre-osteoporotic population. While detailed periodic biochemical assays are currently being performed, major outcomes will be available upon completion of the study period. This group has also pioneered some of the estrogen and supplemental calcium studies. Another investigator at Texas Women's University recently began follow-up x-ray and biochemical evaluations on subjects who had been analyzed 20-25 years earlier. The objective is to compare bone density and biochemical results to age, dietary, and other factors. An epidemiologic project has just begun at the Mayo Clinic to determine the natural history of bone loss especially as reflected by the incidence of femoral and vertebral fractures. Another grantee at Pittsburgh University is utilizing modern CT scanning technology to measure bone loss in individuals with various life styles with the primary goal of assessing the effect of exercise on osteoporosis prevalence. A study on the etiology of osteoporosis is underway at Indiana University. These investigators are measuring bone density in diabetic patients to substantiate the preliminary evidence of excessive bone loss in that group of patients.

A grantee from Washington University (St. Louis) has performed a clinical study using a pulse dosage of sodium fluoride in combination with calcium and vitamin D. Preliminary results showed a reduction in symptoms during a 16-month evaluation period. This group also evaluated osteoporotics treated with calcitonin. Despite a decrease in serum calcium, there was no significant effect on bone density. A recently initiated study at the University of Washington (Seattle) is searching for a factor that may couple bone formation and resorption. If successfully identified, this agent may be the controlling substance whose function is altered in osteoporotic patients.

The Musculoskeletal Diseases Program released a Request for Applications on a Clinical Trial of Fluoride in Osteoporosis. The resulting grant (to be funded in FY 81) will evaluate the safety and efficacy of this currently controversial therapeutic agent. Correlative studies on the basic biological metabolism of fluoride were included.

Several other bone diseases are being investigated. Examples are the viral etiology of Paget's disease, vitamin D resistant osteomalacias, and a clinical trial of several new metabolites of vitamin D in the treatment of bone defects in children with renal osteodystrophy.

Research interest in heritable connective tissue disorders has expanded during the past few years. An investigator from Johns Hopkins University is studying the genetic heterogeneity in osteogenesis imperfecta. Many factors will be

analyzed to define more clearly the several phenotypes of this disease. Researchers are also studying samples of diseased tissue to identify the biochemical or physical nature of the defects. A grantee at Jefferson Medical College (Philadelphia) is utilizing light and electron microscopic histomorphometry to evaluate tissue samples from patients with osteogenesis imperfecta. Preliminary findings show a primitive collagenous amorphous bone matrix and some spherical structures of unknown organic nature. At Children's Hospital Medical Center (Boston) investigators have found reduced hydroxylation of the $\alpha 2$ collagen chain in Ehlers-Danlos Syndrome patients. Several defects have been noted in both quantity and quality of the various collagen types. In addition, a researcher at Case Western Reserve University has perfected a chondrocyte culture system that allows the cells to maintain their normal biochemical function in vitro. Proteoglycan synthesis rates and subsequent aggregation were reduced in the first patient studied.

One facinating study by grantees at Johns Hopkins and Washington University (St. Louis) resulted in a successful marrow transplant from a sibling on a five-month old osteopetrosis patient. This normally fatal disease now has a potentially successful treatment, if a proper allogenic donor can be obtained.

Osteoarthritis

Research grants in the area of osteoarthritis include both biomechanical and biochemical research activity. The biomechanical studies measure and model forces, geometry, and lubrication of normal and diseased joints. An investigator at Massachusetts Institute of Technology has utilized ultrasonic transducers to observe the bone geometry and cartilage thickness in unloaded cadaver joints. In the loaded condition, several pressure maximums were found to correspond with bony irregularities beneath a spherical cartilage surface. A recently initiated study at Pittsburgh University will attempt to measure pressure distribution with miniature piezoresistive stress transducers in cadaver joints. Other grantees are applying tiny strain gages to the bone underlying articular joints and telemetering information from a walking horse. Investigators at the Cleveland Clinic have modelled contact areas and pressure distributions in the hip and ankle joints. The results indicate that the ankle transmits force more efficiently than the hip.

Several studies are concentrating on characterizing the properties and lubrication of articular cartilage. At Beth Israel Hospital (Boston) grantees measured viscoelastic properties for use in a hip joint model. The results of predicted trabecular bone area and orientation compared favorably with actual anatomical measurements of bone cross-sections. Recently, a large grant has been awarded to Rensselaer Polytechnic Institute to undertake a multi-phasic study of biochemical, mechanical, and electromechanical phenomena in normal, biochemically modified, and experimentally induced osteoarthritic cartilage. Investigators at the University of California, San Diego, are measuring cartilage-cartilage interfacial shear with a new testing apparatus. Results show a dependence of shear on load, load history, and ionic balance, but no correlation with velocity. A study at Massachusetts General Hospital has identified a specific glycoprotein (LGP-I) primarily responsible for lubrication. Additional tests are now being performed to observe the structure, synthesis, and age related changes in these molecules.

Two interesting studies are concerned with the transport of molecules into cartilage. One researcher is utilizing radioactive tracers to measure movement of various sized molecules into joint tissue. Another study at Pittsburgh University is measuring and isolating joint wear products by magnetic separation with Ferrography. Several unique particles have been found that suggest both rubbing and cutting wear mechanisms.

Several basic biochemical studies deal with both the components and the regulating agents in the articular cartilage. Investigators at Massachusetts General Hospital have shown that collagen synthesis initially increases in arthritic tissue and later decreases, as the severity exceeds a critical point. Similar correlations have been made regarding the activity of proteoglycan components. A Tufts University grantee has shown that increased levels of dietary vitamin C reduced the incidence of osteoarthritis in a joint trauma model in rats. The same investigator demonstrated the presence of a proteoglycan enzyme (arylsulphatase) in normal chondrocyte cultures. Other grantees are studying the regulation of proteoglycan aggregates by lysozymes. Finally, two grant projects have developed relationships between chondrocyte sensitivity to prostaglandin E2 and the subsequent onset of osteoarthritic fibrillation.

The artificial joint section of the Musculoskeletal Skin Diseases Program is closely related to the osteoarthritis portion. Many of the mathematical analyses serve as useful models in either the normal or artifical joint area of research. Some of the scientific areas covered include biomaterials, fixation, systemic effects, failure analysis, joint resurfacing, and instrumented endoprostheses.

Fracture Healing

Projects supported in fracture healing cover a spectrum of basic and applied science. A grantee from the Hospital for Special Surgery is studying the basic biochemical changes in various phases of the healing process. The changes in matrix composition resemble endochondral ossification in the normal growth plate. At the Mayo Clinic, investigators demonstrated that transcapillary transport in bone occurs by passive diffusion. With regard to bone infection, antibiotics have been shown to reach cortical bone at effective levels. Other studies indicate that medullary blood flow is the dominant source of supply in healing bone.

A clinically oriented study at the University of California at San Diego is developing an optimized intermedullary rod for fixation in complex fracture situations. The new device resists bending and twisting but allows the bone to support more normal forces. The result is a reduced bone resorption from stress shielding. At Louisiana State University, investigators are developing a non-invasive device that utilizes the piezoelectric properties of bone to detect the extent of healing more accurately and safely than X-rays.

A major new approach to the clinical management of fractures is developing rapidly. The Food and Drug Administration has recently approved three electric stimulation devices for limited use in cases of non-union, failed fusion, and pseudoarthrosis. Investigators at the University of Pennsylvania use a transcutaneous cathode at the fracture site and a skin anode several inches away. A multi-stranded distributed cathode wire has been found to be more effective

than a single wire. Some ultrastructure studies display an initial invasion by polymorphic cells and calcification preceding the presence of osteoblasts. Other results showed a decrease in pCO_2 and pO_2 levels near the cathode. A second device utilizing external electromagmetic stimulation coils is being studied at Columbia University with some dramatic successes in patient trials. In canine experiments, results show: (1) There are similar healing patterns as with fixation plates. (2) The sham operations are unsuccessful. (3) Minimal weight-bearing is essential. (4) An increase of alkaline phosphatase occurs in gap tissue chondrocytes. (5) There is a general absence of matrix vesicles at the site of calcification. These investigators are also experimenting with other applications for the pulsed electromagnetic device.

Two projects, supported at Battelle Laboratory, relate to the basic phenomena of inducing bone healing with electric stimulation. In vivo experiments were performed in rabbit calvaria using direct current techniques. Increases have been noted in calcium, proline, and hydroxyproline levels, as compared to controls. Cyclic AMP activity has been too variable to assess. Some of the experiments with different electrodes demonstrate the importance of the effects of an electric field, rather than the simple occurrence of gross electrochemical reaction. In vitro studies are progressing with a clonal cell line from a rat osteosarcome. After 24 hours of direct current stimulation at 100 μ amperes, there is a two to four fold increase in cyclic AMP levels at the cathode. Alkaline phosphatase activity and collagen synthesis are decreased, as compared to controls.

MULTIPURPOSE ARTHRITIS CENTERS PROGRAM

Overview

The Multipurpose Arthritis Centers Program is currently at a level of twenty-four Centers located in eighteen states. Fifteen Centers were first funded in FY 1977, seven more in FY 1978, and two in FY 1979. In October 1979, seventeen applications were received for review for funding in FY 1980. Fifteen of these were from the Centers first funded in FY 1977, and two came from institutions which do not presently have Arthritis Centers. The applicant institutions were all visited by teams of experts, which conducted the initial review. The resulting reports were taken to the NAMDD Advisory Council for a final recommendation.

In FY 1977, $2.5 million was available for the Arthritis Centers program. The fifteen funded Centers received an average of $167,000 (total costs) ranging from $61,000 to $200,000 for the initial year. For FY 1978, $5.2 million was available and the average award to a Center was $234,063. During FY 1979, when a total of $6.2 million was expended on Center activities, the average size of an Arthritis Center was approximately $275,000.

Activities in the Centers

The Arthritis Centers conduct activities in three areas: basic and/or clinical research, educational activities, and community demonstration projects. The new projects now underway could not have been started without the interaction of individuals brought together under the aegis and support of the Arthritis Center grants. The funds first awarded for the program in FY 1977 were used predominantly for educational and community demonstration component activities, as recommended in the Arthritis Plan. Subsequently, increased support has been given for innovative and developmental research projects which supplement the traditional investigator-initiated research grants associated with each Center. This research encompasses a wide range of disciplines and interests from biomedical to health services research.

Research Component Activities

As a prerequisite for award, each Arthritis Center is expected to possess a substantial base of ongoing basic and/or clinical biomedical research in areas related to the rheumatic diseases. These activities are not supported by Center grant funds, but rather through traditional research grant mechanisms. Center funds are used, however, to support developmental and feasibility studies in rheumatology-related areas of biomedical research. These studies are an important feature of the Multipurpose Arthritis Centers Program in that they encourage investigators to explore interdisciplinary and highly innovative scientific approaches which may later form the basis of regular research grant applications.

At the University of Alabama Center in Birmingham, researchers are attempting to identify changes that occur in articular cartilage at the onset of degenerative joint disease (osteoarthritis). The study is focusing on the immunological detection of changes in cartilage proteins using a highly sensitive

radioimmunoassay on a strain of mice which is predisposed to develop degenerative joint disease. Once the assay system has been perfected using this animal model of osteoarthritis, samples of human articular cartilage will be analyzed to see if changes in human cartilage parallel those in animal tissue.

In related investigations, the Arthritis Center at the Medical College of Wisconsin is examining collagen biosynthesis in experimentally induced osteoarthritis. Although previous studies suggest that cartilage lacks the ability for self repair, Center researchers have found that this hypothesis may be incorrect. Using a model of surgically-induced osteoarthritis in the rabbit knee, investigators noted that regenerating cartilage can be observed as early as one week after surgery. By ten weeks after surgery, the induced defects have been repaired by a type of collagen that is biochemically unique to cartilage. This study is significant in demonstrating the ability of cartilage to regenerate itself and to produce a cartilage characteristic of the original articular cartilage--a situation previously not thought possible.

An innovative method for the study of rheumatoid arthritis was developed at the Dartmouth Arthritis Center in Hanover, New Hampshire. Cells from the diseased joint lining (synovium) of patients with rheumatoid arthritis were injected into a special strain of mice to study the invasive property of these cells. This strain of mouse (athymic "nude" mice) has previously been used in cancer and basic immunological research. The Dartmouth researchers found that after 21 days, tissue at the injection sites resembled to a remarkable extent the pannus (diseased tissue) of human rheumatoid arthritis. The tissue contained many fibroblasts, multinucleated cells, blood vessels and bundles of collagen fibers. Tissue-typing showed that the tissue was of human origin. Of special interest was this tissue's ability to produce collagenase and prostaglandin E2, a feature of diseased joint lining in rheumatoid arthritis in man. These investigations will provide a new method to study the factors that influence the proliferative lesion of rheumatoid arthritis.

Another study of potential importance is being conducted at the Washington University Arthritis Center in St. Louis, Missouri. Researchers are ascertaining the effects of glucocorticoid therapy on bone mineral metabolism. A complication of such therapy is the development of accelerated bone loss, a process which leads to a markedly increased incidence of bone fracture and chronic, disabling bone pain. More than two million persons are at risk for this condition in the United States alone. The etiology of steroid-induced bone disease and the factors which predispose to developing osteopenia remain to be defined. Using the results of high pressure liquid chromatography analyses, Center researchers have obtained initial data suggesting that steroid administration induces production of abnormal vitamin D metabolites in animals and man. These metabolites could potentially interfere with the action of normal vitamin D metabolites or could have direct, deleterious effects on bone metabolism. The information gained from these studies may ultimately lead to improved methods for the prevention and treatment of glucocorticoid-induced bone loss in humans.

In addition to basic research, clinical trials are underway at several Centers. At Stanford University, a trial, carried out by both academic and community rheumatologists, was conducted to determine the efficacy of six non-steroidal anti-inflammatory agents in treating rheumatoid arthritis and ankylosing spondylitis (spinal arthritis). Two of the drugs were superior to the others in treating ankylosing spondylitis, while in rheumatoid arthritis, all of the drugs were pharmacologically equivalent. A new study has been planned to compare the efficacy of different treatments in scleroderma.

In a novel project conducted at the University of Missouri Center in Columbia, scientists are investigating whether a computer-based system can serve as a useful clinical consultant for the non-rheumatologist physician. At present, five versions of the computer model can make the correct clinical diagnosis from 82% to 92% of the time, when compared to the consensus reached by a panel of clinicians. The work is significant because rheumatological expertise, at the consultant level, is often not available to patients suffering from arthritis and related musculoskeletal diseases. This "automated consultant" approach, therefore, may provide a means of imparting the knowledge possessed by specialists in rheumatology to large numbers of physicians who are not experts in this field.

Education Component Activities

A major effort in the Arthritis Centers continues to be educational activities. Many ongoing projects are intended to improve rheumatic disease care at the level of the primary care physician through studies designed to identify and/or develop effective training approaches. For example, at the Robert Breck Brigham Hospital in Boston, a Center physician who specializes in a particular group of diseases conducts on-site visits to the office of a primary care physician (PCP). Prior to the visit, the specialist reviews the charts of patients of the PCP in his area of expertise. The PCP and the specialist examine patients together and discuss problems concerning the specific care of that patient and the general capability of the PCP and his office to render specialized care.

In another project directed toward community physicians, the University of Alabama Arthritis Center is continuing its Dial-Access System (DAS). The DAS is a method of medical consultation by means of tape-recorded messages that are accessible on request through long-distance toll-free telephone calls. The DAS makes available, without charge, the most recent diagnostic and therapeutic information on specific arthritis and rheumatology problems. Physicians use the service by telephone, requesting any of the topics listed in the catalog. Almost 1,500 calls per month were made to the DAS during the first part of the year by physicians in various states all over the country.

Another innovative way to educate health professionals has been under development for the past two years at the University of Arizona Center. Center staff noted that physical examination of the joints was often poorly taught in medical schools, because it was frequently carried out by physicians who lacked skill in joint examination. The Center postulated that it might be useful to employ patient instructors: individuals with stable abnormalities

of the musculoskeletal system, who had been trained to evaluate the quality of student skills. To date, over 100 medical students have been instructed and evaluated by these patient instructors. The data thus far indicate that through this method of instruction, preclinical medical students have become as adept at examining arthritis patients as their clinical seniors. The program is so successful that it has been fully integrated into the physical diagnosis courses at the University.

Community Component Activities

Community component projects during the past year have included studies of the social and economic impact of arthritis, model demonstrations of care in non-Center locations, and to increase the awareness of the general population concerning the problems of arthritis.

At the University of Alabama Center, a prospective study is measuring the impact of rheumatoid arthritis. In its initial stage, the study has focused on design of an instrument to reliably assess the costs of the illness and the functional and activity limitations it imposes. This instrument can then be employed to evaluate outcomes of arthritis treatment in the future. An initial group of 73 patients has been admitted to the study.

The cost-effectiveness of joint replacement is being studied at the Cornell University Center. At its Hospital for Special Surgery, rheumatoid arthritis patients are evaluated according to vocational, economic and functional parameters, both before and after surgical rehabilitation. Following surgery, a cost analysis also will be performed, including assessment of hospital expenses, rehabilitation services, physicians' fees, and outpatient costs, along with plans for periodic followups over several years.

Arthritis in Children

Many of the Multipurpose Arthritis Centers are active in pediatric rheumatology. The Center at the University of California in San Francisco has regular clinic conferences devoted to the presentation of pediatric rheumatologic problems. Similar clinics are being held at the Dartmouth Center involving the concept of multidisciplinary team care, including input from the family physician, pediatrician, rheumatologist, arthritis nurse-specialist, physical therapist, orthopedist, social worker, community nurse, opthalmologist, and others. This program will also conduct epidemiologic studies and analyze the clinical management of children with arthritis throughout the entire region served by the Center. The Stanford University Center is operating a program to determine the psychosocial and functional outcome in persons who have had juvenile rheumatoid arthritis or childhood-onset ankylosing spondylitis. Thus far, 250 patients are participating in the study. When completed, this investigation should help to provide important prognostic data on juvenile-onset patients whose disease persists into adulthood.

Coordination Among Multipurpose Arthritis Centers

A special emphasis of the NIAMDD program management is to foster and effect inter-Center activity in order to make the Multipurpose Arthritis Centers

Program more efficient. This is done through the sponsorship of annual meetings of the Center Directors, compilation of a Directory of Center personnel, production of an index of audiovisual materials produced by the Centers, and the sharing of educational facilities.

SPECIAL PROGRAMS IN ARTHRITIS AND RELATED DISEASES

The following important activities are being developed to address certain specific features of the National Arthritis Acts of 1974 and 1976, and several recommendations of the Arthritis Plan submitted to the Congress in April 1976 by the National Commission on Arthritis and Related Musculoskeletal Diseases.

EDUCATION PROGRAMS

The NIAMDD Education Programs Office is concerned with serving the information needs of the health professional in order to achieve optimal treatment and management of arthritis.

Arthritis Information Clearinghouse Office

A major component of the Education Programs Office is the Arthritis Information Clearinghouse. Established by the NIAMDD following authorization by the National Arthritis Act and recommendation by the National Commission on Arthritis and Related Musculoskeletal Diseases, the Clearinghouse is completing its second year of operation aimed at improving the lines of communication among the health professionals serving the arthritis patient. The major function of the Clearinghouse is to collect, screen, store, and disseminate information about educational materials and programs on the rheumatic diseases.

In serving as a broker to foster the flow of arthritis information, thus helping users to locate and select educational materials, the Clearinghouse refers clients to the appropriate developer or source rather than acting as a distributor of the materials itself.

The Clearinghouse data base is now fully operational, consisting of 1800 records, retrievable by keyword, author, source, publication year, and text words in titles and abstracts. Included in the data base are print and audiovisual materials, journal articles, reports and textbooks, among other items.

Products and Services

Clearinghouse products thus far include 25 bibliographies and catalogues, a Thesaurus, and a cumulative catalogue of 1979 accessions, while its services, approximately 300 per month, include requests for publications, mail list additions, data base sources and referrals.

Due to a heavy demand requested by users for literature searches, the data base has grown rapidly. In order to streamline requests, packaged bibliographies on frequently requested topics have been prepared. To speed answers to information and referral questions, several directories have been assembled. A total of 15,542 Clearinghouse products have been distributed during the past fiscal year.

As part of its outreach program, four Arthritis Information Clearinghouse Bulletins have been published this year to alert users and health professional constituents as to new developments. In addition, two of the Clearinghouse exhibitis have been sent to meetings and conferences, including the annual

meetings of the American Rheumatism Association and the Arthritis Research and Education in Nursing and Allied Health.

Cooperation with Multipurpose Arthritis Centers

The Education Programs Office has initiated a project to collect information on community and institutional patient education and in-service training programs relating to arthritis. Since many of the Multipurpose Arthritis Centers are emphasizing team care and developing programs and resources to implement education programs, there was a need to develop a method for the Clearinghouse to identify MAC efforts in this area and incorporate knowledge into the Clearinghouse data base.

A number of possible alternatives have been explored to strengthen communication between the Clearinghouse and the MACs. For example, the Clearinghouse should be a depository for MAC reports, reprints, and studies. Curricula and program descriptions of patient education or in-service training classes are being collected, and materials developed such as syllabi, resource lists and supplementary materials are also being sought.

In the next fiscal year, the Clearinghouse will continue to provide direction and assistance to education activities within the centers program, and will continue to conduct needs assessments and to identify gaps in available materials. Since both the Clearinghouse and the MACs exist through NIAMDD funding, Clearinghouse users assume a close communication link between the two programs to improve the accessibility of educational materials to patients and health care providers.

Advisory Group

An advisory group comprised of eleven rheumatologists, educators, library scientists and other health professionals has continued to help the Clearinghouse in the effective accomplishment of goals. The group, meeting twice yearly, acts as a sounding board and provides recommendations to the Clearinghouse project officer and staff.

Appraisal and User Satisfaction

Inasmuch as the bibliographic information services have matured and become operational, and a host of products and services have been produced after two years of operation, it had been evident that an evaluation or appraisal was needed to determine the effectiveness of the program. To help evaluate sevices, users have been asked to return a User Satisfaction Form enclosed with mailed materials. Most users have found services very helpful and are satisfied with the response times. In addition, unsolicited letters have been received which relate that the Clearinghouse products, services, and activities have saved physicians, health educators and other professionals considerable time and effort in educating patients and other health professionals.

ARTHRITIS DATA SYSTEMS PROGRAM

The Data Program Office is organized to foster the systematic acquisition, storage, retrieval, and analysis of information about arthritis and related musculoskeletal diseases. Its concerns include insuring validity, confidentiality, and comparability of data collected in separate institutions and integrating data sources and data needs so that they would be most useful to the largest number of persons. Two working groups assist the Program Office and the Arthritis Interagency Coordinating Committee Data Systems Subcommittee.

This past year a joint NAAB/NIAMDD committee reviewed efforts by the National Center for Health Statistics (NCHS) to collect and analyze data on the rheumatic diseases based on the Health Examination Survey and Health and Nutrition Examination Survey (HES/HANES). Resulting recommendations will be reviewed by the Data Systems Subcommittee which will explore alternative means for implementing these recommendations. To facilitate review of HES/HANES, an inventory of NCHS data collection and analysis was produced by the Data Systems Program Office. Other inventories of non-government and government population-based collections are in progress.

ARAMIS

The American Rheumatism Association Medical Information System (ARAMIS), supported by the Institute, is the prototype for a national arthritis data system. The ARAMIS community currently includes 12 geographically dispersed members, each with their own separately maintained data bank at Stanford. More than 14,000 patient records are included in the various banks. Patients once entered are the subject of continued long-term investigation. Presently the average is 20 visits per patient.

Using the Time Oriented Database system (TOD), ARAMIS is able to display longitudinal patient data trends over time. This software approach is unique and provides for inverted storage of masses of data which can be readily accessed for covariable and other analyses. After a thorough review of alternatives, TOD was selected recently by NINCDS for use in their Stroke and Coma Data Banks.

The following is a sampling of projects at the various ARAMIS centers: (1) a search for sensitive prognostic predictors in progressive systemic sclerosis; (2) explorations as to the statistical distortions resulting from omitted data in the databank; (3) cost effectiveness of various diagnostic measures in the evaluation of patients with arthritic symptoms; (4) the cost/benefit of total hip replacement in different disease and age groups; (5) the variability between clinics of laboratory results for selected commonly performed tests; (6) a clinical comparison of results with non-steroidal antiinflammatory drugs; (7) an analysis of ARA diagnostic criteria for rheumatoid arthritis; and (8) an attempt to determine the most sensitive prognostic variables for juvenile chronic arthritis.

ARTHRITIS EPIDEMIOLOGY PROGRAM

The Epidemiology Program Office provides stimulation and support to an area where very limited work has been done in the past decade. In addition, the office is responsible for facilitating cooperation and exchange of information among independent investigators, both government and nongovernment.

A major thrust of this year's program has been to organize an international conference on epidemiology in the rheumatic diseases. The objectives are: (1) to convene an interdisciplinary group of rheumatologists and epidemiologists who will review the current state of epidemiologic knowledge of the rheumatic diseases and define gaps in that knowledge, (2) to identify research opportunities, propose research strategies and determine required resources, (3) to focus attention on and attract researchers to the investigation of the rheumatic diseases through the discipline of epidemiology.

An annotated bibliography, prepared in conjunction with the conference, will serve as a base of information to conference participants. Publication of the bibliography as well as conference proceedings and an in-depth article are scheduled to maximize exposure to potential researchers.

This past year a survey of epidemiologic studies in the Multipurpose Arthritis Centers was completed. It is part of a continuing process of identifying promising epidemiologic studies which might provide a model for other investigators to consider.

Associate Director's Report for the
Annual Report of the Digestive Diseases and Nutrition Cluster

The Digestive Diseases Program has been responding to the recommendations of the National Commission on Digestive Diseases. Steps have been taken to initiate a National Digestive Diseases Education and Information Clearinghouse. A committee of experts from within and outside the Government have suggested guidelines which provide a basis for the program which is now being developed. This will include preparation and distribution of fact sheets on specific digestive diseases for the public and patient. There will also be a publication of information on recent research findings and recent activities with respect to digestive diseases both within and outside the Government. Informational and educational materials that are currently available through private and public sources will be assessed and catalogued, so that those interested can be informed about them.

Under the auspices of the Digestive Diseases Coordinating Committee and with the logistical support of the Fogarty International Center, a group has been formed to standardize terminology and diagnostic criteria in digestive diseases. It will begin with disorders of the colon.

In an effort to obtain more epidemiological information on digestive diseases, NIAMDD is cooperating with the National Center for Health Statistics. It is providing ultrasound equipment to be used in the National Health Survey to identify patients with gallstones. Information will also be obtained on the symptoms of the patients and on possible causative factors. Other initiatives in epidemiology are under study with the Epidemiology Section of the Digestive Diseases Coordinating Committee.

Functional bowel disorders were highlighted by the Commission and were the subject of a workshop that considered the psychological, social, motor and endocrine factors that may be associated with this disorder. Workshops were also held in other areas identified by the Commission as appropriate for expansion of research. Topics included gastric emptying, experimental ulcers produced by behavioral factors, endocrine aspects of digestive diseases, genetics, and heterogeneity of gastrointestinal diseases, immunologically mediated hepatotoxicity and the etiology of pancreatitis.

Many procedures used in the study and diagnosis of patients with digestive diseases involve biopsies or instrumental procedures for which there are little or no data on risk. Yet such data is needed in reviewing research proposals and/or clinical trials which use the procedure. Since 1975, the Intersociety Committee on Human Experimentation in Digestive Diseases has been meeting to consider and develop ways of gathering data on the risks of different types of procedures used in diagnosis and treatment of digestive complaints. We now are arranging to bring representatives of the Society together with representatives of federal agencies concerned with human risk, to address the problems of gathering data for quantitating risk and the risk/benefit ratios. Following this meeting, we plan a workshop to develop protocols for gathering needed data on risk.

A consensus conference on the use of endoscopy in upper gastrointestinal hemorrhage is planned for August 1980. On the first two days of the meeting, a series of experts will present the relevant data and discuss the key issues with panel members responsible for developing a statement of consensus. These issues include the benefits of endoscopy, the risks of the procedure, and the alternatives to endoscopy in upper GI bleeding. On the third day of the conference, the panel, chaired by Sherman M. Mellinkoff, M.D., Dean of the School of Medicine, UCLA, will present its consensus statement and invite comments from the audience which should include interested experts.

The Nutrition Program has expanded its translational activities and its teaching materials with the help of Dr. Wayne Callaway, a phyisician who specializes in clinical nutrition and has served with the program on IPA. The number of clinical nutrition research units has been expanded by the addition of two new units. Trace metal deficiencies has been the subject of two workshops.

DIGESTIVE DISEASES PROGRAM AREA

(1979-1980)

The Digestive Diseases Program Area is responsible for the extramural support of grant awards and contracts pertaining to certain organ systems of the gastrointestinal tract. Digestive Diseases include disorders of the esophagus, stomach, and small and large intestines. Major digestive diseases include esophagitis, peptic ulcer, diverticulitis, infectious diarrhea, ileitis, ulcerative colitis, and malabsorption. The known causes included infections, metabolic disorders, genetic defects, neuromuscular disorders, toxins, and reactions to the life stress.

Digestive Diseases constitute a health problem of great magnitude. Of all the causes of disability due to illness in our country, digestive diseases rank second. Over two million Americans are impaired in some degree by the disease; of these 1.2 million are limited in performing useful work and 400,000 are totally disabled. Among the employed, digestive diseases each day account for 200,000 absentees from work due to illness, making it the leading cause of lost time from work among male employees. Almost 140,000 veterans receive payments for service connected disability due to gastrointestinal conditions, at a cost to the nation approximating 100 million dollars per year.

During the period of October 1, 1979 through September 30, 1980 approximately 192 research grants, one specialized center of research, 17 training programs, 28 career awards, and 15 individual fellowships have been supported in the Digestive Diseases Program Area. In addition, several contractor efforts were also active which included a clinical trial and applied research and development projects.

A. Research Activity Supported By Grants

During the FY 1980 much of the research activity supported by grants was concerned with the basic science aspects of digestive diseases. A greater part of the total research, however, was disease oriented, the results of which were relevant to the elucidation and understanding of the diseases process. Of the considerable amount of data and information reported by the grantees during the past year, only a few examples will be cited as representative of the progress that has been made in the Digestive Diseases Program Area.

1. Peptic Ulcer Disease

a. Prostaglandins are now ubiquitous in the body and play an important regulatory role in many physiological and pathophysiological processes. The special role of prostaglandins in protecting the mucosa of the stomach and intestine is now recognized. All of the many drugs which inhibit the synthesis of prostaglandins, including aspirin and the many aspirin-like drugs in clinical use, produce injury to the gastric

mucosa and this side effect is a major problem in the clinical use of these drugs. It has been established beyond reasonable doubt that chronic ingestion of aspirin causes not only acute erosions but also chronic gastric ulcer. Administration of exogenous prostaglandins protects the gastric mucosa against injury by a wide variety of injurious agents as diverse as ethanol and boiling water. The exact mechanism by which prostaglandins exert this protective action is not known. Although prostaglandins inhibit acid secretion, this is not the main mechanism of protection since they are effective in doses that do not inhibit acid and some prostaglandins that do not inhibit acid also protect. There are suggestions that the protection may involve stimulation of blood flow or stimulation of secretion of mucus and bicarbonate by surface epithelial cells. Elucidation of the mechanism by which prostaglandins protect against mucosal injury is a major goal of research in this area.

Stimulation of endogenous production of prostaglandins by mild injury of the gastric mucosa protects it against injury by stronger damaging agents. There is evidence to suggest that alteration of prostaglandin production may be involved in such diverse mucosal diseases as peptic ulcer, stress ulcers, small intestinal ulcers, pseudomembranous colitis, ulcerative colitis, and perhaps others. As a general principle, it appears that prostaglandins play a role in protecting gastric and intestinal mucosa against injury from noxious agents in the lumen. These noxious agents may be of endogenous origin such as acid, pepsin, bile acids, lysolecithin, bacterial toxins, and perhaps others. Or they may be of exogenous origin such as drugs which inhibit synthesis of prostaglandins, alcohol, and perhaps others. Preliminary clinical trials show that prostaglandins are effective in the treatment of mucosal lesions produced by drugs which inhibit prostaglandin synthesis and peptic ulcer.

b. Genetic studies are playing an important role in our increasing awareness of the heterogeneity of the ulcer syndrome. Genetic approaches include: the delineation of rare inherited disorders that feature peptic ulcer as part of their phenotype, clinical genetic studies that involve detailed analysis of the phenotype of the ulcer patient, and genetic physiologic studies using subclinical markers. Each of these approaches has already contributed to our knowledge of peptic ulcer genetic heterogeneity, yet much more remains to be done in each of these areas. The existence of rare genetic syndromes that feature peptic ulceration is an important demonstration of genetic and etiologic heterogeneity. Besides suggesting heterogeneity in "common" peptic ulcer, the study of these disorders serves the more important function of elucidating different pathogenetic mechanisms that can lead to the ulcer diathesis. Clinical genetic studies are playing a central role in understanding peptic ulcer genetics by providing the first definitive separation of gastric and duodenal ulcers.

Such studies also suggest that combined gastric and duodenal ulcer may be a separate disorder. Additional clinical studies indicate that childhood duodenal ulcer may be a distinct entity. The association of

of a common defect with pleiotropic manifestations as has been suggested for chronic pulmonary disease and lung cancer, renal stones without hyperparathyroidism, and possibly coronary artery disease.

Clinical studies are being conducted utilizing subclinical markers which are closer to the underlying defect and will detect more individuals with the abnormal genotype. Rather than looking for a single defect in all ulcer patients, the biochemical and physiologic differences between patients will be utilized as clues to delineate different disorders. This approach has been used most extensively with the subclinical marker serum pepsinogen I (PG I). It has been shown in family, sibling, and twin studies that duodenal ulcer patients are either hyper PG I or normo PG I on a familial basis, thus providing definitive evidence of genetic heterogeneity of "common" duodenal ulcer. Furthermore, each of these groups are being studied and subdivided by further physiologic studies, such as the gastrin response to a protein meal and the rate of gastric emptying. Thus, accumulating evidence suggests that there are multiple forms of peptic ulcer, each with a different genetic basis. Based on the currently available evidence a preliminary genetic classification will be obtained.

2. Inflammatory Bowel Disease

a. Crohn's disease is a chronic inflammatory bowel disease of the small and large intestine whose etiology is unknown. The nature of the anatomic lesions and the clinical course of the disease suggest an infectious etiology. A variety of agents including viruses, L-form of bacteria, and mycobacteria have been inconsistently isolated from tissues of patients with Crohn's disease. Current research studies have demonstrated a putative viral agent obtained from mesenteric lymph node filtrates from patients with Crohn's disease. There filtrates from patients with Crohn's disease. These filtrates produduce B cell lymphomas in athymic nu/nu mice whereas similar filtrates from control patients did not. Filtrates of these lymphomas produce similar lesions in a second group of nu/nu mice. Antigenic recognition of the lymphoma occurred with all sera of patients with symptomatic Crohn's disease before and after absorbtion by mouse Ig using indirect immunofluorescence. This did not occur with sera from asymptomatic Crohn's disease patients, those with ulcerative colitis, and noninflammatory bowel disease. Cells and cell-free filtrates of lymphoma cultures produced splenomegaly or generalized lymphadenopathy in nu/nu mice. Spleen cells from these mice were passaged as many as ten times in culture and continue to show positive immunofluorescent staining. These studies strongly suggest that lymphoma production in nu/nu mice by Crohn's disease tissue filtrates is related to a transmissible agent which is also present in murine lymphoma. At present, the relationship of the agent to the active disease in man is speculative and awaits clarification from continued research studies.

b. Regardless of the primary etiologic agent or factor, evidence is available to suggest that certain humoral and cellular immunologic

processes play a part in the persistence or chronicity of inflammatory bowel disease. These disorders are often included in the category of autoimmune diseases. Included among the immunologic aberrations found in patients with Crohn's disease or ulcerative colitis are high titers of antibodies to colonic epithelial cells and the apparent cytotoxicity of circulating lymphocytes for these cells. Cytotoxicity, or cell killing reflects mechanisms involved in cell-mediated immune responses which includes the white blood cells. Certain of these, termed natural killer cells, will spontaneuously kill a variety of other living cells. This process is suggested to be mediated by receptors on the surface of the killer cells. Recent studies have been reported which indicated that endogenous lectins, cell membrane molecules composed of sugars and proteins, may be important elements in allowing the white cells to recognize and kill other cells. These sugars, among others, include ribose, lactose, arabinose, mannose, and sorbose. It was found that these simple sugars, in experimental preparations, were able to inhibit spontaneous killing by natural killer cells, perhaps, by binding to the white blood cells surface receptors. Previous studies have shown that the protein degrading enzyme trypsin also inhibits natural killer cell activity. These findings suggest that the surface receptors involved in interactions between natural killers and target cells are endogenous elucidating the complex mechanisms involved in cell-mediated immune responses and specifically, the process of cytotoxicity. By using these sugars as probes, it may be possible to better understand the killing process, the cells which mediate killing, the mechanisms involved, and how to control the destruction of body cells, specifically, the intestinal epithelial cells.

B. Research Activities Supported by Contract

1. National Cooperative Crohn's Disease

Contract No. NO1-AM-2-2210

University of Colorado, Denver, Colorado

Total Duration 5 years

Total cost $1,915,262

Crohn's disease (regional enteritis) causes severe abdominal pain, diarrhea, malnutrition and sometimes death. Many patients have prolonged periods of hospitalization, and sometimes abdominal surgery, involving the removal of portions of the small intestine, is necessary.

The results of the long term study, now completed, show that both prednisone and sulfasalazine are significantly better than a placebo in causing a remission of active Crohn's disease. Azathioprine was somewhat better than a placebo, but not enough to be statistically significant. None of the three drugs was better than a placebo in preventing flareups or reoccurrence of the disease.

A second phase of the study questioned whether prednisone and sulfasalazine together might be better than prednisone alone. The combination of both drugs was compared to using prednisone alone in 89 patients. The study showed that the combination, if anything, was less effective than prednisone alone for inducing remission.

In the course of screening more than 1,400 patients and studying over 700 of them for periods of up to two years, the investigators were able to make other determinations about Crohn's disease. For example, the x-ray characteristics of the disease were carefully and exhaustively defined to aid radiologists in recognizing and following its course. In addition, the value of rectal biopsy in diagnosis was examined and found to be minimal. The response of Crohn's disease to surgery was also documented to aid surgeons in deciding when to operate on patients with the disease.

The complete results of the NIAMDD cooperative study were reported in the journal, Gastroenterology, Volume 77, Number 4, Part 2.

This study was conducted under the direction of Drs. John Singleton and Fred Kern, Jr., of the University of Colorado.

2. Research and Development Studies in Gastrointestinal Endoscopy

Contract N01-AM-5-2211

Title: Endoscopic Control of Continued Massive Gastric and Duodenal Bleeding in a General Hospital Population

Institution: University of Washington, Seattle, Washington

Duration: Four years

Contract Amount: $872,066

This contract study is being supported to study the control of gastrointestinal bleeding by endoscopic systems involving laser coagulation, electrocoagulation, suture clips, and tissue glues. To date, significant progress has been made. Biocompatible tissue glues and suture clips have failed to stop bleeding in the ulcer model that was developed during the early phases of the contract. Only one modality has been found to be promising for the safe and effective control of massive bleeding. This is the argon laser used in conjunction with pressurized carbon dioxide. Currently, a pilot controlled clinical trial for approximately 100 patients at the University of Washington is being conducted. The data obtained from this study will serve as the basis for a larger multicenter controlled trial.

The studies at the University of Washington are under the direction of Drs Fred Silverstein and Cyrus Rubin.

C. WORKSHOPS

The Digestive Diseases Program Area supported five workshops on conferences during FY 1980. The primary objectives of these meetings were to review the state-of-the-art, identify priority research needs, and to mutually educate the clinical and basic scientists.

1. Experimental Ulcers Produced by Behavioral Factors

October 20-21, 1979

SUMMARY: The meeting was devoted to review and discussion of the environmental and behavioral factors and neural mechanisms that may influence or be responsible for the formation of gastric and/or duodenal lesions (ulcers). More specifically; the topics that were covered during the symposium included: 1) factors in gastric mucosal resistance to injury, 2) brain-gut relationships, 3) human peptic ulcer disease, 4) production of lesions by behavioral factors, 5) opportunities and needs for future research.

2. Functional Disorders of the G.I. Tract - October 25-27, 1979

SUMMARY: This meeting brought together scientists and investigators with multi-discipline backgrounds and interests. Primarily, they included gastroenterologists, pediatricians, physiologists, behavioral specialists, epidemiologists, and experts in motility disorders of the gastrointestinal tract. The topics that were reviewed and discussed intensively included: 1) survey of common clinical disorders, such as disorders of swallowing, biliary dyskinesia, anorectal disorders, and irritable bowel syndrome. 2) behavioral aspects including epidemiological studies, sociologic factors, behavioral analysis, learning theory and reward systems, biofeedback, and psychophysiological studies, 3) control mechanisms including distribution of peptides in brain and gut and their loci of action, roles of neurotransmitters, myogenic mechanisms and 4) opportunities for research.

3. Cell Basis of Digestive System Chemical Messengers - January 17-19, 1980

SUMMARY: An International Symposium on the Cellular Basis of Chemical Messengers in the Digestive System was successfully conducted. The participants included twenty-nine persons from nine countries. The emphasis of the symposium was on the structure and ultrastructure of the endocrine cells and nerves of the digestive system which produce the amines and peptides which serve as chemical messengers regulating the activity of this organ system. Much new information on the localization and distribution of amines and peptides in specific endocrine and neural cells was presented. An update of the Classification of Gastroenteropancreatic Cells, last revised at a meeting in Lausanne in 1977, was prepared and will be published as a part of the proceedings which will be a volume in the UCLA Forum in Medical Sciences.

4. Gastric Emptying - March 3-4, 1980

SUMMARY: The meeting was devoted to discussion of current ideas about control of gastric emptying of both liquid and solid foods. Topics included the roles of the proximal and distal stomach, the localization and characteristics of intestinal receptors controlling gastric emptying, type of gastric smooth muscle contractions and electrical phenomena and the role of nerves and hormones in the control of gastric emptying. Also methods for measuring gastric emptying were extensively reviewed. Also discussed were the problems of identifying the subgroups within the population; findings in a family with duodenal ulcer and rapid gastric emptying were presented. Four groups of participants discussed abnormally slow gastric emptying in diabetes and other conditions, as well as their results with trials of metaclopramide.

5. Genetics and Heterogeneity of G.I. Diseases - March 17-19, 1980

SUMMARY: Emphasis was placed on common gastrointestinal disorders including peptic ulcer, atrophic gastritis, lactose intolerance, celiac disease, inflammatory bowel disease, gastrointestinal cancer, gallbladder disease, GI malformations, mathematical genetic approaches, genetic marker association studies, and gene environment interactions. The state of our current knowledge of the genetics of many of the common GI disorders was clearly delineated.

The presentations and ensuing discussions made it clear that genetics could usefully interact in advancing all phases of our knowledge in gastroenterology. Not only is it important to determine the risks of recurrence of disorders within families, it is apparent that genetics is an extremely powerful tool to dissect out the pathophysiology and natural history of a disorder, and in the process demonstrate that there exist several disorders which were thought to be one. It can also be used to identify individuals for prophylactic therapy.

The proceedings of the meeting will be published in a volume by Academic Press and should serve as a valuable reference for our knowledge of the genetics of these disorders and a starting point for future studies.

LIVER DISEASES PROGRAM AREA

1979 - 1980

Overview

During the past year, the Liver Diseases Program area completed a clinical trial on fuminant hepatic failure; held two important research conferences; phased out one of the two comprehensive Liver Centers and established a Core Liver Center in its place; instituted an interagency agreement with DRR to revitalize a flock of mutant Corriedale sheep to serve as a model of Dubin-Johnson syndrome; and initiated a recurrence study under contract as a continuation effort of the National Cooperative Gallstone Study. The largest area supported by the program continues to be the individual research grant program, accounting for about 84% of total expenditures. Since this program has not grown in size since last year, a program announcement was made, aimed at attracting basic scientists and clinical investigators from other areas into liver research. One new institutional training grant was added but one was terminated with the total remaining at 14. The individual fellowship program and the career program remained modest in size, a cause for some concern because of the nationwide decrease in clinicians entering into a research career.

Although only a few items will be highlighted, research supported by the program encompasses the broad area of liver diseases, including basic physiology, metabolism and morphology. Certain of the diseases, such as viral hepatitis and schistosomiasis, neonatal jaundice and biliary atresia, alcoholic hepatitis and liver cancer, are supported primarily by other Institutes (NIAID, NICHD, NIAAA and NCI, respectively). The NIAMDD supports research on the acute and chronic liver diseases whether of viral, drug or environmental toxin origin when the focus is on the disease and its progression rather than on the etiology. Basic studies are supported on collagen formation and degradation as it relates to liver fibrosis and cirrhosis; etiology and therapy of primary biliary cirrhosis; Wilson's disease; hepatic coma and portal hypertension. Bile pigment metabolism and its relationship to jaundice, Gilberts', Dubin-Johnson and Crigler-Najjar syndrome and hepatic porphyria; as well as studies on liver regeneration, liver transplantation and liver support devices are also included in the program. The morphologic liver abnormalities of Reye's syndrome, fatty liver, alcoholic hyaline are part of the program.

Highlights of research activities of grant-supported investigators will be found in the first section of the following report. Results of the clinical trial on fulminant hepatic failure, description of two workshops and a progress report on the continuing and new phase of the National Cooperative Gallstone contract will be found in succeeding sections.

I. Research and Center Grant Activity

A. Transport in Liver (Univ. Calif., San Francisco Liver Center)

Three investigators, two of whom are still in a training status, have made significant contributions to the understanding of varying transport functions of the liver. A. McDonagh has elucidated the mechanism whereby phototherapy of the jaundiced neonate allows unconjugated bilirubin to be excreted into the bile. The normal glucuronide conjugation of bilirubin is poorly developed in these jaundiced infants. Phototherapy appears to work by changing a rigid, highly hydrogen-bonded isomer of bilirubin which cannot be excreted into bile without conjugation, into a labile form in which the hydrogen bonds are broken, giving the molecule the flexibility to be secreted into the bile without conjugation. Once in the bile, it reverts back to its rigid form and is not reabsorbed, with the net effect that unconjugated bilirubin is excreted.

R. Weisiger, a trainee, has studies to suggest that the liver has albumin receptors and that the albumin is an important determinant in the uptake of long-chain fatty acids. Until now, it has always been assumed that the albumin merely transported the fatty-acid to the liver, where the complex dissociated. It now appears that some undissociated complex attaches to the albumin receptors. The fatty acid is then bound to receptors and transported into the liver from the plasma.

R. Renston, a Ph.D. candidate, has electron microscopic evidence to show immunoglobulin A (IgA) being taken up by the liver from plasma and being secreted by hepatocytes into the bile. The IgA lacks its "secretory component" when it is taken up from the plasma, and the liver adds the "secretory component" before excretion into the bile. Therefore, a fraction of intestinal IgA may be entering from the bile after Goblet cells in the lamina propria of the gut secrete IgA into the portal circulation. It has always been assumed that only the gut was capable of adding "secretory component" and that all the IgA found in the gut came there via direct secretion from the Goblet cells of the gut.

B. Chronic liver disease (Albert Einstein, N.Y. Liver Center)

Only a small percentage of patients who have an acute attack of viral hepatitis go on to develop chronic liver disease, cirrhosis and possibly even liver cancer. Current tests for viral antigens and antibodies in the serum or liver, as well as studies of liver morphology have been unable to distinguish those who will be likely to develop chronic disease. D. Shafritz has now increased the sensitivity of a test for detecting viral particles by 1000 times over the previously most sensitive technique of radioimmunoassay for HB_sAg and has thereby greatly increased the possibility of detecting the persistence of the virus in the liver. He has accomplished this feat by using the newest techniques in molecular biology - cloning of the Hepatitis B virus and examining small samples of the liver directly for the presence of viral DNA. Such DNA can be detected even if the virus is not being produced in the liver.

C. Hepatic Coma (Cornell Univ., New York)

The controversy about the etiology of the coma resulting from severe

liver disease continues to rage, with ammonia, mercaptans and other toxic metabolites still vying for the dubious honor. The focus of attention has now properly shifted from the liver to the brain and neurologist T. Duffy is using the stable isotope ^{13}N to label ammonia to look at its metabolism and distribution in normal individuals versus patients with liver disease and encephalopathy. In humans, it has been difficult to show a consistent pattern for brain ammonia metabolism by measuring arterial and cerebral venous concentrations. The failure to demonstrate that ammonia enters the brain has been cited as a weakness in the hypothesis that hyperammonemia produces coma. Duffy's findings using quantitative body scans show that arterial ammonia is taken up and used by the brains of all subjects and that the rate of utilization increases linearly with arterial ammonia concentration. Subjects with hepatic encephalopathy also had higher arterial ammonia concentrations. About 50% of the ammonia cleared from the blood is taken up by skeletal muscle, the most important organ in ammonia homeostasis in patients with advanced liver disease and portal-systemic shunting in contrast to the liver (urea cycle) which is most important in normal individuals. Severe muscle wasting in these patients was found to be associated with a worse prognosis for survival than any of the other more usual indicators of severe liver disease.

D. Albumin decrease in severe liver disease (Mass. Gen. Hosp., Boston)

The decrease in serum albumin in severe alcohol-induced liver disease (ALD) has always been ascribed to an impaired synthetic function of the liver. Now J. Wands and J. Dienstag have demonstrated an exciting new finding; i.e., that the serum albumin is low because of circulating autoantibodies to native albumin which ties up the albumin as immune complexes. Immunoglobulin synthesis was strikingly increased in peripheral blood mononuclear cells from patients with ALD. In addition, binding activity for albumin was detected in 12/12 patients with ALD but in only 1/20 patients with chronic active hepatitis or primary biliary cirrhosis.

II. Completed Clinical Trial on Fulminant Hepatic Failure

To evaluate the effectiveness of corticosteroid therapy in acute hepatic failure of viral or drug etiology, the Institute sponsored a 4 year randomized, controlled clinical trial which was terminated in 1979. Specialists in liver disease at research facilities throughout the United States and Canada collected standarized information concerning cases they saw and carried out cooperative investigations of the management of acute hepatic failure. Although hydrocortisone has been used since 1951 in acute hepatic failure, it had not been established whether it had a beneficial or detrimental effect. This cooperative study showed neither benefit nor harm, but an "incidental" finding provided a clue to the possible explanation of the discrepancies among previous unblinded studies. Thirty-five percent of patients survived when the cause of hepatic necrosis was one of the two known hepatitis viruses (A or B) whereas only seven percent survived when the cause was a non-A, non-B virus or a drug. The marked effect of etiology on survival

therefore, must be taken into account in any conclusions about the influence of specific therapies.

III. Workshops

A workshop on the "Regulation of Hepatic Cholesterol and Bile Acid Metabolism", held at the NIH on October 19-21, 1979 achieved its objective of encouraging the exchange of ideas between those scientists interested primarily in blood lipids and those interested primarily in liver lipids. Approximately 80 people, including prominent scientists in the field and young investigators participated in the meeting. A small workshop dealing with "Issues in Immunologically Mediated Hepatic Injury", held at Scripps Clinic and Research Institute in La Jolla, California Jan. 29, Feb. 1, 1980 brought together basic immunologists with hepatologists. In addition to bridging the gap between the basic and clinical scientists, it offered an opportunity for 20 young investigators in the field to interact with the 20 distinguished speakers.

IV. National Cooperative Gallstone Study Contract
NO1-AM-3-2216
Cedars-Sinai Medical Center
FY 79 - $1,423,591

This multi-center, randomized controlled clinical trial has been underway since 1973, although the clinical phase was not started until September 1975. The study is evaluating the safety and efficacy of chenodeoxycholic acid administered at two dosages compared with placebo over a two year period in 1000 carefully selected patients with cholesterol gallstones. All of the patients will complete this main study by September 1980 and the results of the study will be analyzed and written up for publication during the following year.

A new phase of study (termed Phase III or recurrence study) was just initiated during the past year although it had been an aim of the original protocol. This study compares a low dose with placebo as a means of preventing recurrence of gallstones in that group of patients whose gallstones dissolved in the preceding study. This group of patients will be treated for at least two years with either 375 mg/kg chenodeoxycholic acid or placebo. They will be withdrawn from treatment in September 1982. The results of this study should be of value in deciding about the prophylactic use of this agent in a population at high risk of developing gallstones such as American Indian females. It should also give us information on whether the drug will need to be taken indefinitely to prevent recurrence of gallstones that have dissolved.

NIAMDD ANNUAL REPORT FY 80

NUTRITION PROGRAM - DDN

NIAMDD's Nutrition Program continued its extramural support of basic, clinical and behavioral research in nutrition directed toward enlarging the knowledge needed to improve human health and well being. Nutrition also constituted a significant component of research supported by several other programs, especially Digestive Diseases, Diabetes, Metabolism, Hematology, Endocrinology and Liver Diseases Programs. The FY 80 budget for the Nutrition Program amounted to 14.9 million dollars.

The Institute awarded its first three clinical nutrition research units (CNRU's) in FY 79 as part of a joint effort with NCI and NAI to strengthen multidisciplinary research, education and service activities in clinical human nutrition in health and disease. Two more will be funded by NIAMDD in FY 80. These core grants are designed to provide a focus for clinical nutrition research and related activities in biomedical institutions and complement on-going funded project grants and training awards. The CNRU program represents an innovative approach which is expected to be of considerable value in stimulating progress in multidisciplinary research in clinical nutrition.

The Nutrition Program has continued to stress the following nutrition priorities: research on obesity with the aim toward obesity prevention and control; supportive nutrition for hospitalized patients; human nutritional requirements and factors that influence these requirements; new technologies in the study of the function of nutrients; iron deficiency; metabolic roles of trace minerals; dietary fiber; interaction of nutrients and nutrition and immune competence.

Research by Contract

The ninth revision of the "Recommended Dietary Allowances" was completed under contract to the Food and Nutrition Board, National Research Council, National Academy of Sciences, and distributed in February 1980. This revision of the RDA's contains tentative levels for fluorine, copper, manganese, molybdenum, selenium and chromium. Under this contract, the Food and Nutrition Board also completed a report on "Research Needs for Establishing Dietary Guidelines for the US Population". This contract has been renewed to 1) initiate the preparation of the 10th edition of the RDA's (to be completed in 1981), 2) prepare guidelines for use of the RDA's and 3) to prepare dietary guidelines for specific life-cycle sub groups based on an interpretation and translation of research data relating to diet and health status.

The NIAMDD Nutrition Program continued to administer the US Malnutrition Panel of the US-Japan Cooperative Medical Science Program. Two workshops were held during FY 79 - one at Osaka, Japan on "The Effects of Alterations of Dietary Patterns and Food Habits on Health with Special Reference to Critical Analysis of Comparative Epidemiological Studies of Different

Populations" and one at Bethesda on "Methodologies for Human Population Studies on Nutrition Related to Health". A third workshop will be held July 29-31 in Morioka, Japan on "Effects of Nutrition on Human Growth and Development and on Later Life and Health: Global Priority Issues". NIAMDD funded seven grants totaling $540,221 or slightly more than half of the 1.074 million support for this program in FY 79.

RESEARCH GRANTS - RECENT TRENDS

Nutrition, Neurotransmitters and Behavior

Studies have shown that certain dietary nutrients affect the levels of precursors of neurotransmitters in the brain, and in turn the amount of neurotransmitters formed. The amino acids, tryptophane and tyrosine, are required to form the neurotransmitters, seratonin and dopamine, respectively. Choline is needed for the synthesis of acetyl choline.

Research at the University of Wisconsin (Harper) and MIT (Wurtman) has revealed that the ratio of serum tryptophane or tyrosine to other large neutral amino acids (LNAA) in the serum correlates best to rates of uptake in the brain. Not only does the level of each of these amino acids eaten at a meal in relation to the amounts of other LNAA (which compete for transport across the blood brain level), but dietary factors which influence insulin secretion may affect amino acid uptake by the brain. Insulin enhances the rate of uptake of some of the LNAA (leucine, isoleucine and valine) by striated muscle, thus improving the ratio of trytophane or tyrosine to other LNAA at the site of uptake into the brain. Thus carbohydrate tends to favor brain uptake of both tryptophane and tyrosine, which results in the formation of higher levels of serotonin and dopamine in the brain.

The functional significance of increased amounts of neurotransmitters in man is unclear, but observations in animal models and preliminary work with selected patients suggest promise to this approach. Wrutman (MIT) has found that oral tyrosine administration resulted in lowering the blood pressure in spontaneously-hypertensive rats. Oral tyrosine increases serum tyrosine in humans. Its use in Parkinson's Disease, depression and hypertension is being studied. Increased tryptophane and serotonin levels are believed to reduce blood pressure, depression and to increase growth hormone production. Patients with Tardive Dyskinesia appear to respond favorably to 20 gms per day of dietary lecithin which contains choline and increases acetyl choline levels. It appears clear that the potential effect of diet on behavior through neurotransmitters and their function is potentially very important.

Obesity Classification and Thermogensis

Obesity is the most common form of malnutrition in the U.S., reaching a peak in prevelance of 39% in men and 50% in women over age 30 years who 10% or more overweight. The etiology of obesity in most individuals is not known, but its association with other serious chronic diseases (especially diabetes mellitus and hypertension) gives it major public health significance.

The Nutrition Program in FY 80, initiated efforts to develop a physiologically valid and clinically applicable classification of obese states. A work shop has been planned for late spring 1981 on advice from an eight person steering group of selected researchers. The workshop will focus on methodologies which may be used to differentiate obese subjects into specific types for use in therapeutic and preventive intervention studies.

Greenwood (Vassar) has pair-fed obese and lean Zucker rats (offspring from Fa fa female x fa fa male) the amount of food voluntarily consumed by the lean rats. In spite of this food restriction, the obese Zucker rats became equally as fat as ad libitum fed obese controls. Lipoprotein lipase levels were elevated as early as seven days of age in the obese rat model.

During the past year a number of investigators have ephasized differences in heat production or thermogenesis as a factor in obesity.

Romsos (Michigan State University) has found that the Na^+-K^+-ATPase are doubled in both obese and lean mice when exposed to a cold (14^o C) as compared to a warm (33^o C) environment. This finding suggests that cold stimulated thermogenesis is attributed in part to changes in sodium pump activity. The concentration of (3H) ouabian binding sites in skeletal muscle and liver also were doubled. Reduced total binding sites were observed in obese mice, which may account for their reduced energy expenditure.

Danforth and Tulp (University of Vermont) found thermogenesis to be markedly increased in protein-malnourished rats. This was accompanied by increases in serum concentrations of triiodothyronine (T_3) and thyroxine (T_4).

Hashim (Columbia University) has observed increased thermogenesis in rats fed medium-chain fatty acids with less deposition of body fat than in controls fed diets containing comparable amounts of metabolizable energy from long chain fatty acids.

A recent observation of great potential importance, is the finding that brown fat (which has been known but not understood for many years) deposits may be important sites of free energy production. Brown fat cells are very rich in mitochondria and appear to burn up fat and produce heat. Cafeteria fed rats have more brown fat and are leaner than meal fed rats. Cold exposure markedly increases brown fat depots, and animals which hibernate have greater brown fat stores. Infants have brown fat at birth and it is believed to serve as a central heating system.

Infra red photography has been used to show brown fat areas on the back of the necks, back and chest in human subjects. Brown fat is stimulated to produce heat by norepinephrine. This finding suggests that with a better understanding of the effect of diet, exercise, enviroment and drugs on thermogenesis, control of obesity may be within reach.

Zinc, Nutrient Interaction and Immunocompetence

The critical importance of the dietary level of zinc as it may influence the requirement for certain other trace elements and its role in immunocompetence has been demonstrated. The production of metallothionein (Whanger, Oregon State University) by the intestional wall is increased when dietary zinc is increased. At the same time, metallothionein strongly binds copper and iron as well as zinc and cadmium, rendering them less available. In carefully monitored studies low copper levels were observed in volunteers receiving 125 mg of supplemental zinc daily (Prasad). This observation suggested that the ratio of certain minerals in the diet may have practical significance, especially in that zinc supplementation may increase the need for dietary copper.

Studies on immunocompetence by Good (Cornell University) and others has revealed that zinc adequacy has a marked affect on immune function in rats. The practical significance of this finding has been confirmed in children by Golden (University of West Indies), who has reported rapid changes in immune response following zinc supplementation. His work suggests that zinc inadequancies and impaired immunocompetence in man could be expected in protein low diets, since most foods considered to provide important amounts of zinc also are good sources of protein.

An NIAMDD investigator (Anthony, University of Texas) has begun studies to evaluate changes in in vivo immune competence in precisely defined dietary deficiency, and to assess whether changes occur secondary to primary protein-calorie malnutrition.

Another extensive and important study on zinc metabolism in young children (Hambidge, University of Colorado) involved 46 subjects in a double blind study, with 11 mg of zinc administered daily to half of them. Although only a few of the children have completed the study, the results indicate a significant increase in food comsumption and a trend to greater growth velocity in the test children compared with controls.

Studies on the bioavailability of zinc for the human breast milk was 4 times more available than that from cow's milk and 13 times greater than that provided by a low-phenylalanine semi-synthetic diet.

Other Research

Walser and co-workers (Johns Hopkins) has found that the keto analogues of certain essential amino acids (ex. leucine) are more effective in nitrogen retention than are equivalent amounts of the amino acids.

This group has developed new compounds composed of branched chain keto acids and amino acids to treat congential hyperammonemias and portal systemic encephalopathy. These compounds are neutral or sweet in taste and quite acceptable to patients. They can be used to improve protein nutrition and delay dialysis or transplantation in end stage renal disease.

Studies at the Obesity Center, St. Luke's Hospital, NYC, with the recently perfected universal eating monitor have progressed effectively in measuring differences in eating pattersm of obese and lean individuals. Individuals ate fewer calories when a highly palatable diet was modified to contain less energy, suggesting the low-calorie analogues are effective in promoting weight loss. Added fiber has been used with success in certain foods.

Studies on "protein-sparing" low calorie diets for weight loss revealed that a hypocaloric diet consisting only of protein did not spare body nitrogen better than an equicaloric mixture of protein and carbohydrates.

Oscai (University of Illinois) has found that the newborn rat can be programmed by controlling food consumption in early life for a permanently lower or higher voluntary food intake. They also have found that lipoprotein lipase can act as a mobilizing emzyme capable of hydrolyzing endogenous triacylglycerols. The evidence suggests also that this enzyme may be involved in the regulation of tissue triglycerides in the heart.

Results obtained by Anderson (V.A. Hospital Lexington) suggest that high-carbohydrate, high-fiber diets may be the dietary therapy of choice for patients with maturity onset type diabetes mellitus and may have distinct benefit for patients with hyperlipidemia. Long term studies showed that diets containing 50 grams of plant "fiber" per day are well tolerated and show no adverse nutritional effects.

Oace (University of california, Berkeley) has standardized model systems for evaluating the effects of dietary fiber on intestinal transit time, fecal enzymology and the bioavailability of micronutrients. A vitamin B_{12} depleting effect of pectin has been firmly established.

Prasad (Wayne State University) observed decreases in zinc concentrations of plasma, erythrocytes, leukocytes and urine of four male volunteers who received restricted intakes of zinc for several weeks under metabolic conditions. Changes in the activity of zinc dependent enzymes in plasma, such as alkaline phosphatase and ribonuclease were related to zinc status, this providing a basis for developing diagnostic criteria for zinc deficiency in humans.

An improved method of measuring magnesium absorption using stable ^{26}mg as a tracer has been developed by Schwartz (Cornell). Even though the method requires fecal samples within 3-4 days and urine samples within 2-24 hours use of ^{26}mg (the stable isotope) significantly expands the scope of investigation of magnesium absorption in man beyond that possible with the short lived radioisotope ^{28}Mg alone.

Carlisle, UCLA, has found that a dietary deficiency of silicon resulted in abnormal skull formation and a reduction of collagen content in chicks. Such a pronounced effect suggests that knowledge about the role of silicon may be important to the prevention and treatment of connective tissue disorders.

Benevenga, University of Wisconsin, has found that the transaminative pathway of metabolism of methionine degradation is active in the human liver. Already two homocystineric patients have been treated with betaine for over 6 months with marked clinical benefit.

Norman and others at the University of California, Riverside, detected by radioimmunoassay a vitamin D-dependent calcium binding protein in rachitic bone, which differs in molecular weight from the previously identified calcium binding protein in the intestine. It is concentrated principally in the spongy and cartilage plate regions of the bone, and may have important clinical implications.

June 24, 1980

ANNUAL REPORT (1979-1980)

BILIARY TRACT AND PANCREATIC DISEASES PROGRAM

A. Program Mission

A major function of the Biliary Tract and Pancreatic Diseases Program is to support research, both basic and clinical, and related training into the normal function and the causes, diagnosis, prevention, and treatment of the diseases of the biliary tract, the exocrine pancreas, and the salivary glands.

B. Background Information

1. Diseases of the Biliary Tract:

(a) Cholesterol Gallstone Disease. It has been estimated that between 12 and 13 million people in the United States have cholesterol gallstones with all of their human and economic consequences. The advent of a medical treatment (chenodeoxycholic acid) that is effective in dissolving some stones is an advance of enormous importance. However, much more information is needed on the natural history of the long-term therapy. Further, it is generally held that prophylaxis is the ultimate objective, and for this we need a better understanding of the biophysical processes by which lipids are secreted in bile and nucleation occurs so that the pathogenesis of the disease state can be delineated.

(b) Pigment Gallstone Disease. Pigment gallstones, which constitute 24% of all gallstones, affect about 4 million people. Important advances have been made recently in the epidemiology and composition of these stones, but much remains to be done to elucidate the processes controlling pigment secretion, stone nidation and growth, and the role of biliary constituents in preventing precipitation. More basic knowledge is necessary before methods for dissolution and prevention can be devised.

(c) Cholestasis in Adults. Cholestasis, particularly that associated with the administration of a wide variety of medications, is an increasing problem. No effective therapeutic approaches are available, and the differential diagnosis is difficult. Progress will depend on elucidation of the basic mechanisms involved in bile secretion. Much has been learned recently about bile salt-dependent and -independent flow, but the molecular bases, while tantalizingly close, remain a mystery.

(d) Diseases of the Biliary Tract in the Newborn Infant. Treat-

ment of most of these devastating diseases in infants and children is now based on surgical correction of the defect or liver transplantation. While these modalities need further development, the ultimate solution to the problem will be prevention - and this will require knowledge of the pathogenesis. Current knowledge is barely beyond the stage of classification.

2. Diseases of the Pancreas:

Among adults, the commonest diseases of the pancreas, other than cancer, are acute and chronic pancreatitis. Among children, cystic fibrosis is the commonest pancreatic disease, occurring in about 1 in 1,000 births. More than 100,000 persons are hospitalized each year in the U.S. for pancreatic diseases other than cancer. A far greater number who are not hospitalized suffer from pancreatic diseases. Alcoholism is by far the commonest cause of pancreatitis and gallbladder disease is the second most common cause, but in neither case do we understand how these conditions lead to pancreatitis. The enzymes of the pancreas digest food in the intestine. Normally these enzymes do not become active until they reach the intestine. In pancreatitis, the enzymes escape from the pancreatic cells into tissue spaces and become activated by unknown mechanisms. Research aims to find out why these abnormal events take place and how they can be prevented or reversed. The pancreas is a relatively remote organ, so only recently have diagnostic methods been developed which allow full display of its size, shape, and relations with surrounding organs. These new tools require evaluation. Much of our modern information about how cells manufacture proteins and package them for export to the exterior of the cell has been learned from studies in which the pancreatic cell served as a model. Palade won the Nobel prize for this work in 1974. It is important that this fundamental information on the biology of the pancreatic cell be pursued in such a way as to allow its application to the diagnosis, treatment, and prevention of pancreatic disease.

C. Progress Reports

As of April 30, 1980, 3 individual postdoctoral fellowships, 7 career awards (5 research career development awards and 2 clinical investigator awards), 78 research grants, and 1 conference grant were being supported by the Program, reflecting a 16% increase in total activities since last year's annual report, when the corresponding figures were 2, 7 (6 and 1), 68, and 0 respectively. Approximately 64% (57 activities), 30% (27 activities), and 6% (5 activities) of the 89 activities under support were within the biliary tract, pancreas, and salivary gland areas, respectively, representing a subfield distribution almost identical to that in last year's report.

In Fiscal Year 79, 44 applications were assigned to the Program. Eighty-two percent (82%) or 31 of the 38 applications approved were funded.

Although a considerable amount of research and other activities, both basic and clinical, has been either reported by the grantees or initiated by the Program during the past year, only a few examples will be cited to illustrate the progress that has been made in various areas of interest to the Program.

1. Biliary Tract:

(a) In its January 31, 1979 report to the Congress of the United States, the National Commission on Digestive Diseases recommended that the National Center for Health Statistics (NCHS) and the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD) should collaborate in studies to ascertain the prevalence of gallstones in suitable population groups. The NIAMDD and the NCHS, accordingly, have recently entered into an Intraagency Agreement to include an epidemiological study of gallstones in the next two Health and Nutrition Examination Surveys (HANES Surveys). The two surveys, which will last for a total of about 6 years, are a study of Hispanics and the next national survey (HANES III). While the NCHS shall be responsible for the actual conduct of the survey and the collection and analysis of the resulting data, the NIAMDD will collaborate in the development of the design and protocol of the study and will provide support, through reimbursement to the NCHS, for certain specific costs related to the medical aspects of the study. Specifically, the objectives of the subject collaborative agreement on gallstone survey in the United States national and Hispanic populations are: 1. To estimate the prevalence of gallstones and their distribution according to age, sex, and other demographic factors. 2. To determine the correlation between the incidence of gallstones and the incidence of gallbladder disease and morbidity. 3. To determine the effect on gallstone formation of: (a) fasting and, conversely, frequent snacking, (b) caloric intake, (c) dietary cholesterol, (d) vegetable fiber intake, (e) physical exercise, (f) obesity, (g) polyunsaturated fat intake, (h) diabetes, (i) pregnancy, (j) postmenopausal estrogen intake, (k) use of oral contraceptives by women of childbearing age, (l) drug intake history, and combinations of these variables. Association of cholelithiasis with a variety of other measures included in the multiphasic health examination survey of the population will also be looked for. 4. To study the association of symptomatology with gallstones in different age-sex groups. 5. To establish a registry of cases, suspect cases, and normals.

(b) Obese subjects are prone to bile that is supersaturated with cholesterol. During caloric restriction saturation of their gallbladder bile with cholesterol generally does not decrease; indeed, it may increase above the saturation observed during weight maintenance. However, after stable weight is reestablished and significant weight loss has been achieved, bile generally becomes less saturated with cholesterol. Because obese patients often make multiple attempts at weight reduction, it is clear that they belong to a high risk group in forming cholesterol gallstones. Two related studies were carried out at the University of California, San Diego (Dr. Grundy et al., RO1-AM 16667) on obese subjects to investigate effects of bile acid

feeding on biliary lipid metabolism before and during weight reduction. In one study, chenodeoxycholic acid (CDCA), 750 mg/day, was given to 12 obese subjects during weight maintenance (1st month) and during weight reduction (2nd month). In the second study, effects of two bile acid preparations, CDCA and Bilron (containing mostly cholic acid and deoxycholic acid), randomly administered, were compared in another 12 obese subjects undergoing weight reduction. The results show that obese subjects had large pools of bile acids during weight maintenance which decreased on caloric restriction (1,000 kcal/day). CDCA increased pool size only modestly during weight maintenance, from 3,536 $\pm$ 1,267 (SD) mg to 4,735 $\pm$ 1,434 mg. Both CDCA and Bilron markedly reexpanded the contracted pool of bile acids in obese subjects on weight reduction. However, significantly reduced saturation of bile occurred only in those on CDCA and weight reductions, whereas supersaturation was unaltered when weight was maintained constant in these patients, or when Bilron was given during caloric restriction. No significant side effects were noted during bile acid feeding for any of the subjects. Thus, CDCA given to obese subjects on weight reduction will reduce bile saturation and could protect against gallstone formation.

(c) An important step in the formation of bile is the conjugation of the individual bile acids with an amino acid. This reaction is catalyzed by an enzyme present in the soluble fraction of liver and requires the prior activation of the bile acid to a CoA derivative. All animals, except for the placental mammals, conjugate their bile acids exclusively with taurine. However, in certain of the placental mammals, glycine conjugates are also found. The basis for the appearance of glycine conjugation among the placental mammals was investigated at the University of California, San Francisco (Dr. Vessey, RO1 AM19212). The reaction of cholyl-CoA with glycine and taurine, as catalyzed by the soluble fraction from guinea-pig liver, had a high affinity for taurine and a poor affinity for glycine. The predominant synthesis of glycine conjugates in the guinea-pig can be related to the fact that guinea-pig liver contains an unusually low concentration of taurine and a high concentration of glycine. Rabbits make exclusively glycine conjugates, even though their livers contain low concentrations of taurine, because their soluble liver fraction has a high affinity for glycine and a poor affinity for taurine. Alternative-substrate-inhibition studies with glycine and taurine in soluble fractions from guinea-pig and rabbit liver revealed that glycine and taurine were mutually inhibitory, suggesting that there is only one enzyme for glycine and taurine conjugation in these tissues. The soluble fractions from bovine liver and human liver also made both glycine and taurine conjugates and evidence is presented that suggests that there is only one enzyme in these tissues too. Even the rat, which excretes mostly taurine conjugates, could make both glycine and taurine conjugates in vitro. However, in contrast with all of the placental mammals studied, the supernatant fraction from liver of the chicken and other non-mammals could not make glycine conjugates even in the presence of very high concentrations of glycine.

(d) In earlier studies at the Cleveland Clinic Foundation (Dr. Holzbach, RO1-AM17562), it was concluded that biliary cholesterol supersaturation may be a necessary but not sufficient cause for gallstone

formation. In the present studies, cholesterol saturation indices (CSI) were calculated for 120 bile specimens of cholesterol gallstone patients and controls using the solubility boundaries of Carey and Small for artificial biles. The mean CSI $\pm$ SD was 1.42 $\pm$ 0.68 for controls and 1.80 $\pm$ 1.02 for cholesterol gallstone patients. Of the control bile samples, 68% were supersaturated. Since the two groups could not be sharply distinguished by saturation index, another property of bile samples was studied: nucleation time for cholesterol crystal formation (i.e., the time, in days, of incubation at 37°C required to detect by polarizing microscopy the earliest formation of solid cholesterol microcrystals in an isotropic (one phase) sample of bile. The mean nucleation time for 16 control bile samples was 15 days, and a strong correlation (r = 0.84) was found between CSI and nucleation time. Twelve bile samples of cholesterol gallstone patients had a mean nucleation time of 3 days and no correlation between CSI and nucleation time. A discriminant analysis of the data, which took into account both the CSI and the nucleation time for each sample, allowed a sharp distinction between bile samples of cholesterol gallstone patients and controls. This distinction could not have been drawn from studies with artifical biles, since these differ only in their CSI. Nucleation time is, therefore, a key factor in the pathogenesis of cholesterol gallstone disease.

(e) An *in vivo* intestinal perfusion system was used at the Mayo Clinic and the University of California, San Diego (Dr. Hofmann *et al.*, RO1-AM 6908, 16770, and 21506) to study the effects of different bile acids on fluid secretion, mucosal permeability, and mucosal morphology in the rabbit colon. To define the structure - activity relationships of the bile acids, nine unconjugated bile acids were used, varying only in the number (two or three) or position (3,7, or 12 or various combinations) of hydroxy or keto nuclear substituents. Results showed that bile acids with two hydroxy groups in the configuration at the 3, 7 position, 3, 12 position, or 7, 12 position induced fluid secretion, increased mucosal permeability, and produced mucosal damage as assessed by light and scanning electron microscopy and quantitated by DNA loss during perfusion. Replacement of hydroxy groups by keto groups or a change from α to β configuration for the hydroxylic substituent in the 7 position abolished all three activities. Trisubstituted derivatives, whether hydroxy or keto, did not affect fluid secretion and permeability or cause mucosal damage. These studies indicate that, of the major primary and secondary bile acids in man, only deoxycholic and chenodeoxycholic acids alter colonic structure and function in the rabbit. They show further that the cathartic effects of bile acids have specific structural requirements; and they show that bile acid-induced secretion was invariably associated with increased mucosal permeability and epitheliolysis.

(f) Pigment gallstone patients are believed to have normal biliary lipid excretion. In order to measure this and to better understand cholesterol gallstone formation, the kinetics of biliary lipid excretion were studied at the University of Pennsylvania (Drs. Trotman and Soloway, RO1-AM 05462 and 16549) in three patients who had been cholecystectomized for pigment gallstones and the results compared to those previously obtained in patients cholecystectomized for cholesterol gallstones.

Pigment-stone patients had hyperbolic relationships between cholesterol and phospholipid outputs and bile salt output which were similar to those seen in cholesterol-stone patients. However, pigment-stone patients excreted more cholesterol and phospholipid at high bile salt output but approached those levels more gradually than cholesterol-stone patients. As a result, pigment-stone patients produced bile undersaturated with cholesterol at a lower bile salt output than cholesterol-stone patients, and thus they would be less likely to produce supersaturated bile during low bile salt output such as that occurring during an overnight fast. The data suggest that cholesterol-stone patients, in addition to excreting more cholesterol and less bile salts than normals, have a defect in the rate of lipid output in response to decreasing bile salt output.

(g) Cholesterol gallstone formation is increased in association with female sex steroids, particularly estrogens. Estrogen administration increases cholesterol saturation of bile probably through decreasing hepatic secretion of bile acids. The present study at the University of Colorado (Dr. Simon, K04-AM 00347 and R01-AM15851) examined the effect of ethinyl estradiol, a synthetic estrogen, on bile acid transport and its effect on liver surface membrane content of bile acid receptors, lipid structure and composition in rats. Ethinyl estradiol administration decreased taurocholate transport maximum to 56% of control values. Since a major determinant of bile acid transport is the number of putative bile acid carriers, specific ^{14}C-cholic acid binding to liver homogenates and membrane fractions was determined, but found to be unchanged in ethinyl estradiol treated rats. Since carrier proteins consist of polypeptides which span a fluid-like lipid bilayer, and require either rotational or lateral movements during translocation, the lipid structure may become a rate-determining barrier to maximum transport rates. Following ethinyl estradiol treatment, membrane viscosity is increased 60% above control. In mammalian cells, the percentage of cholesterol is important in determining viscosity. Both free (30%) and esterified (400%) cholesterol are increased. To further examine the role of lipid structure and composition in regulating bile acid transport, Triton WR-1339, a non-ionic detergent, was administered to ethinyl estradiol treated rats. Bile acid transport maximum, membrane viscosity and lipid structure returned to values similar to control. These correlative changes in lipid structure and function support the hypothesis that changes in membrane lipid composition may alter liver cell function; and, in particular, membrane transport capacities.

2. Pancreas:

(a) On November 18-20, 1979, an NIAMDD-sponsored conference entitled "Frontiers in Pancreatic Physiology - A Workshop" was held in Reston, VA. The purpose of the conference was to bring together a large number of investigators with primary interests in several areas of pancreatic physiology to discuss recent advances in knowledge in this field and to generate ideas for future research. The conference was organized by Drs. Solomon and Grossman, University of California, Los Angeles and by Dr. Williams, University of California, San Francisco (R13-AM 26779). Almost 60 scientists attended the conference and 24 of

them made formal presentations. The remainder made brief presentations and took part in discussion sections. The framework of the conference consisted of the following 6 sessions: (1) Neurohormonal Control of Pancreatic Function: Integrative Aspects. (2) Neurohormonal Control of Pancreatic Function: Identification and Characterization of Receptors. (3) Neurohormonal Control of Pancreatic Function: Stimulus - Secretion Coupling. (4) Pancreatic Enzyme Synthesis, Transport, and Secretion. (5) Pancreatic Bicarbonate and Water Secretion. (6) Pancreatic Growth and Regulation of Digestive Enzyme Synthesis. Important areas identified for future research include: (1) the relative contribution of hormonal and neural mechanisms to the pancreatic enzyme response to meals; (2) the existence and importance of inhibitory mechanisms for regulation of pancreatic secretion; (3) factors which interact with secretin to cause the pancreatic bicarbonate response to meals; (4) continued development of radioimmunoassays for cholecystokinin; (5) the mechanism of feedback regulation of pancreatic secretion by intra-luminal proteases; (6) determination of hormonal factors which regulate cell proliferation in neonatal and adult pancreas; (7) cellular mechanisms which mediate the proliferative response to hormones; and (8) the importance of dietary factors as regulators of pancreatic cell proliferation and the mechanism of changes in digestive enzyme synthesis rates in response to dietary changes. A summary of the conference proceedings has been submitted for publication to Federation Proceedings.

(b) Prostaglandins have been noted to have a "protective" effect against gastrointestinal mucosal injury induced by a wide variety of agents, although possible protective effects of prostaglandins on injury to other tissues have not been reported. Dr. Steer at Beth Israel Hospital, Boston, Mass. (RO1-AM 20302) has tested the effect of prostaglandin E_2 (PGE_2) on acute experimental pancreatitis induced by feeding young female mice a choline-deficient ethionine-supplemented (CDE) diet for 24 hr. Administration of 0.05-0.20 μg PGE_2/g body wt 1 hr before and 4 hr after institution of the CDE diet lowered the mortality rate of diet-induced pancreatitis from 56% to 31%. Larger and smaller doses of PGE_2 were without effect. Administration of PGE_2 (0.10 μg/g body weight) diminished the rise in in-vitro LDH (lactate dehydrogenase) discharge and the increase in "free" Cathepsin D activity which occur during diet-induced pancreatitis. Similarly, PGE_2 (0.10 μg/g body wt) diminished the magnitude of the increase in in-vitro protein discharge and the elevated concentrations of trypsinogen and chymotrypsinogen in pancreas fragments taken from mice given the CDE diet. These findings indicate that PGE_2 has a protective effect against CDE diet-induced acute experimental pancreatitis. The Cathepsin D (a lysosomal hydrolase) and LDH (an enzyme which is normally present in the cytoplasm and not actually secreted) changes noted during CDE diet-induced pancreatis suggest that this diet may decrease membrane integrity and thus allow these enzymes to leak out of the lysosomes and acinar cell, respectively, during pancreatitis. Although the basis for the protestive effect of PGE_2 remains unclear, these observations suggest that the prostaglandin may act to reduce the alteration in membrane integrity which occurs during CDE-diet induced pancreatitis.

(c) Dr. Grossman et al. (RO1-AM 20971) at the University of California, Los Angeles, have studied the ability of gastrointestinal hormones to promote growth and changes in composition of the exocrine pancreas in rats. Chronic administration of caerulein, a structural analog of the intestinal hormone cholecystokinin, increased pancreatic weight by about 50% after 10 days. Content of proteolytic enzymes was increased fourfold. Secretin, another intestinal hormone, also increased pancreatic weight by 15%. Given together, these two peptides nearly doubled pancreatic weight in only 2 days. Both peptides increase DNA synthesis and the number of cells in the pancreas, in addition to increasing the size of cells. Part of the increase in cell size was due to large increases in cellular content of enzymes; after 10 days of treatment, proteolytic enzyme content was increased 10 times, lipase content 4 times, and amylase 2 times. The effects of these peptides may be responsible for the maintenance of normal pancreatic size and function since they are able to reverse the loss of pancreatic size and enzyme content which occurs during starvation. An important question to be answered is whether or not these peptides regulate tumor growth in the pancreas in the same way as they regulate growth of normal tissue.

(d) The pancreatic cell secretes approximately 20 enzymes and zymogens in response to stimulation by cholinergic agents (acetylcholine, carbamylcholine) and peptide hormones (pancreozymin, caerulein, gastrin). Stimulation in vitro with both cholinergic and peptide secretagogues results in a marked rise in cellular cyclic GMP. A rise in cytoplasmic free calcium is generally thought to be necessary for the final secretory event, but the source of increased cytosolic calcium, whether from intracellular sources, extracellular sources, or both, remains undetermined. The in vitro system of guinea pig pancreatic lobules has been used by Dr. Scheele (RO1-AM18532) at the Rockefeller University to define the requirements for intra- and extracellular calcium during cholinergic and hormonal stimulation. Secretagogue-stimulated discharge of secretory protein could be divided into two components depending on its response to calcium added to the incubation medium. Component I of secretagogue-stimulated discharge was (a) independent of calcium added to the incubation medium, (b) operative during early periods of discharge, 10 to 20 min (c) responsible during optimal stimulation for the discharge of 6 to 10% of secretory proteins contained in pancreatic lobules, and (d) inhibited after partial depletion (~55%) of intracellular calcium stores by a 30 min preincubation of lobules in calcium-free medium. Component II of stimulated discharge was (a) dependent on calcium added to the incubation medium, increasing proportionately with log increases in calcium concentation, (b) operative continuously throughout the period of stimulation, and (c) responsible during 240 min of optimal stimulation for the discharge of an additional 44% of secretory protein contained in lobules. Component II of stimulated discharge was specific for calcium since Mg^{2+}, Sr^{2+}, or Ba^{2+} did not substitute and was inhibited in the presence of extracellular calcium by the calcium-displacing agents Co^{2+}, Ni^{2+}, Mn^{2+}, and verapamil. In contrast to the secretory response, the tissue response of cyclic GMP remained largely intact to secretagogue stimulation following the withdrawal of calcium from the intracellular space, extracellular space, or both.

3. Salivary Glands:

(a) Several lines of evidence have indicated that the chronic administration of reserpine to rats induces exocrine gland alterations which resemble those of cystic fibrosis (CF). Submaxillary saliva from the treated rats had elevated Na+, Ca++, protein and carbohydrate concentrations and, significantly, had a marked cilioinhibitory effect comparable to that of CF fluids. In view of these observations, the retrograde perfusion assay in the rat parotid gland was used by Dr. Martinez (RO1-AM 18150) at the University of Missouri, Columbia, MO. to investigate the effects of salivary secretions from control and reserpine treated rats on Na+ reabsorption. Results demonstrated that submaxillary saliva from the treated animals caused a 69% inhibition of Na+ reabsorption, accompanied by a 28% reduction in the volume of saliva secreted and a 22% reduction in flow rate. By contrast, submaxillary saliva from control rats caused a 6% inhibition of Na+ reabsorption, a 6% reduction in volume and a 5% reduction in flow rate. Parotid saliva from reserpine treated rats also inhibited Na+ reabsorption to the extent of 39% and caused a 38% reduction in volume and a 33% reduction in flow. Parotid saliva from control rats only inhibited Na+ reabsorption to the extent of 2.7% and caused a 4 - 6% reduction in salivary volumes and flow rates. The inhibition of Na+ reabsorption and the reduction in salivary volume and flow rates caused by submaxillary saliva of reserpine treated rats were either abolished or significantly reduced when the saliva was previously heated to 100° C, frozen and then thawed or kept in glass tubes at 4°C for 24 hours. These results indicate that saliva from reserpine treated rats has comparable effects in this assay system to those of saliva from CF patients and further support its use as an animal model for this disease.

KIDNEY UROLOGIC AND BLOOD DISEASES PROGRAM
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

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The Kidney, Urologic, and Blood Diseases Program of the National Institute of Arthritis, Metabolism and Digestive Diseases continues its emphasis upon support of research into the fundamental mechanisms of biologic processes and diseases, along with emphasis upon clinical research and treatment in the relevant disciplines of nephrology, urology and hematology and their related disorders. The major support mechanism is the investigator-initiated research grant, with more modest and diminishing investment in research contracts. Both formal and informal coordination of areas of overlapping and complementary interests is maintained with other institutes of NIH, other agencies, outside groups, and scientists in the field. Continuing assessment and evaluation of research and program productivity is maintained by program staff and with consultation with outside experts as needed.

Utilizing recommendations of the study, Research Needs in Nephrology and Urology, sponsored by the NIAMDD and completed in 1978, the program has continued its efforts: in expansion of knowledge through the investigator-initiated research grants; to encourage scientists to submit grant applications in the areas relevant to chronic renal disease, its causes, complications, and treatment through a Program Announcement; by sponsoring and planning conferences where cross-disciplinary research needs encouragement; by contract awards for methodological developments; to increase the data base about incidence and prevalence of kidney and urinary tract diseases; and to collaborate with other agencies concerned with problems of end-stage renal disease. An Evaluation of Research in Hematology was initiated by NIAMDD and a contract awarded to Dr. Ernest Jaffe of the Albert Einstein School of Medicine. It is the aim of this study to assess the state-of-the-art and to indicate the potential areas where research efforts can and should be encouraged.

The five Specialized Centers of Research (SCORs) in Urolithiasis which were funded in September 1977 have shown a considerable degree of cross-disciplinary collaboration and productivity. All of the SCOR centers have been site-visited by staff at least once since their initiation. The annual SCOR directors meetings have featured presentations of research and lively discussions among the groups in order to facilitate cooperative research efforts when possible. An evaluation of the SCORs, both for their success in encouraging research in the area of urolithiasis and as a mechanism for stimulating research in an area which requires cross-disciplinary efforts, is in its incipient phase. The productivity of the SCORs in urolithiasis is attested to by the fact that a third of the papers on the program of the Fourth International Urolithiasis Research Symposium, held in June 1980, were presented by investigators supported by SCORs.

The effort to increase the data about Kidney and Urinary Tract incidence and prevalence continues in a modest fashion. The Kaiser Foundation study of Renal Disease and Prevalence Factors has been completed. The data generated have been analyzed and papers are being written for publication. The data from the National Center for Health Statistics data which has been analyzed is in manuscript form. The study of data from the three military services is being analyzed in collaboration with the Uniformed Services University for Health Sciences.

The effort to develop iron chelators for treatment of patients with Cooley's anemia continues by both contract and by conferences to interest new investigators in the field. Efforts to encourage investigators to collaborate across disciplines in the area of toxicology and environmental hazards include joint sponsorship with NIEHS of a conference on Nephrotoxic Mechanisms in Drugs and the Environment.

Coordination and cooperation with other institutes and government agencies continues, largely on an informal basis, but with formal membership on the Coordinating Committee for Blood Related Activities and on the End-Stage Renal Disease Subcommittee of the National Center for Health Care Technology.

HEMATOLOGY PROGRAM

The Hematology Program of the National Institute of Arthritis, Metabolism and Digestive Diseases supports extramural investigation into basic mechanisms of normal blood cell function and disease pathogenesis, and ranges through development of treatment modalities to clinical application and evaluation of treatment of relevant diseases. This research increases both fundamental and applied knowledge about blood. Further, the availability of blood and blood cells facilitates study of membrane phenomena, protein structure and function, etc. to obtain information broadly applicable across the spectrum of medical problems in areas such as chronic renal insufficiency, transplantation, and malignancy.

Major hematologic categories supported are:

1. anemias of genetic origin, particularly the hemoglobinopathies, with emphasis on regulation of hemoglobin and hemoglobin synthesis (e.g., Cooley's anemia, sickle cell anemia).

2. metabolic disorders, related to iron transport and storage (hemosiderosis), porphyrin metabolism (porphyrias), and enzymatic defects (e.g., red cell glucose-6-phosphate dehydrogenase deficiency).

3. nutritional anemias, especially those involving iron, folic acid, and vitamin B_{12}.

4. disorders of blood cell production, such as those encountered in systemic and/or metabolic disorders. Examples are the anemia of chronic renal failure, aplastic anemia, and polycythemia.

5. immunology, of hematopoietic tissue transplantation and of autoimmune hematologic disease.

The Hematology Program budget for the support of extramural research and training represents slightly more than 6.3 percent of the total NIAMDD extramural budget for Fiscal Year 1980, as compared to 6.2 percent the previous year, continuing a modest recovery in funding from that of the lean years of FY 1975-1978.

Anemias of Genetic Origin

The genetic regulation of hemoglobin synthesis continues as one of the most active areas of investigation supported by this program. Aberrant hemoglobin synthesis represents a suitable model for the study of genetic disease in general. This system could provide information directly applicable to the treatment, cure, or even prevention of hematologic disorders such as sickle cell anemia and β thalassemia.

Many investigators supported by the program have made intensive use of the new technology associated with recombinant DNA. As a result, considerable progress has been made toward the identification of the nucleotide sequence of normal human globin RNAs and DNA, as well as in the analysis and comparison of the structures of a variety of mutant hemoglobins leading to such diseases as β globin mRNA has been determined, as well as that of the major portion of the 3'-untranslated region of human globin mRNA. In addition, the nucleotide sequence analysis of the translated portion of human α globin mRNA is nearly complete. Progress in understanding the molecular biology of thalassemia has been made in a number of areas: demonstration of heterogeneity and variability in the amount of β globin mRNA in β^0 thalassemia; demonstration, in homozygous Hb lepore, of deficiency of $\omega\delta\beta$ globin chain synthesis in this syndrome, demonstration of deficiency of β to α globin mRNA in purified heterozygous thalassemia bone marrow cells that is similar in magnitude to that present in peripheral blood cells of the same individuals; and measurement of β to α globin mRNA content in thalassemic bone marrow cells separated according to their stage of erythroid differentiation. A polymorphism in globin gene DNA with exceptionally high linkage to the sickle gene has been identified. Their finding has led to an extremely sensitive and safe method for prenatal diagnosis of sickle cell anemia. The test is useful in approximately 70 percent of pregnancies at risk, where the linkage provides precise definition. The method is unique, since it requires the use of no fetal blood, as prior methods did, and relies only on obtaining anmiotic fluid.

The level of research activity in the field of hemoglobin research continues high. It is anticipated that the nature of the defect, in thalassemia will make it the first molecular disease to be characterized

completely and the first to be cured by genetic engineering. If this does occur, it will be because of the intense research interest in hemoglobin as a model protein.

Metabolic Disorders

An increased knowledge of the regulation of heme biosynthesis would contribute significantly to the understanding of the pathophysiology of several erythroid and hepatic disorders. One group of these, the porphyrias, are the subject of several grants in this program. A new enzyme-free intermediate in the porphyrinogen biosynthetic pathway, pre-uroporphyrinogen, has been discovered and has been shown to be the long-sought substrate for the enzyme cosynthetase in forming uroporphyrinogen III, the precursor of heme. This substrate is the key to a hitherto-unsuspected pathway. Its discovery has major significance for the understanding of the control of heme synthesis in vivo. It should be of considerable use in designing physiologic therapies for the porphyrias.

A key enzyme regulating glycolysis in human red blood cells, phosphofructokinase (PFK) has been shown to be present in several chromatographically distinguishable isozymic forms, each presumably coded by separate genetic loci. An inherited muscle disease associated with PFK deficiency is characterized by absence of this enzyme in muscle, and by half normal activity in the red cells. Recent studies supported by this program have shown that red cell PFK is composed of two nonidentical subunits specific for erythrocytes. The genetic heterogenicity of erythrocyte PFK may explain the reduction in the erythrocyte enzyme activity by approximately 50 percent observed in inherited PFK deficiency associated with myopathy. If this syndrome is due to complete lack of the muscle-type PFK, the residual erythrocyte enzyme is expected to be liver-type. Such a condition has been found in an individual with this syndrome.

Nutritional Anemias

The Hematology Program is actively involved in several important areas of research in nutritional anemias. However, there are many special problem areas which could benefit from an increased effort. Iron, folic acid, and vitamin B_{12} comprise the major nutrients under investigation.

It has been recognized for many years that iron absorption is affected by the status of body iron stores and by the rate of erythropoiesis. An iron-deficient diet, blood loss, induction of hemolysis, and stimulation of erythropoiesis are each factors which can enhance iron absorption.

Similarly, it is known that iron-deficient animals absorb increased amounts of divalent metals, such as cobalt, lead, zinc, and manganese. Recent studies have shown that while an iron-deficient diet will produce a marked increase in the absorption of the other divalent metals, the other stimuli which enhance iron absorption have little or no effect upon the absorption of the divalent metals. This suggests that the effects of an iron-deficient diet and of factors such as phlebotomy have

different effects upon the pathway of iron absorption in the mucosal cell. Ultrastructural studies in combination with radioautography and benzidine staining of heme are being used to delineate the absorptive pathway of hemoglobin iron in an effort to clarify the differential control factors.

A simplified lymphocyte deoxyuridine suppression test has been developed, in which the amount of blood required has been reduced from 50-100 ml. to only 1-2 ml. The essential feature of the test is the suppression of the incorporation into DNA of radioactive thymidine by exogenous deoxyuridine. Using the test, it has been shown that resting lymphocytes have virtually no uptake of folate, vitamin B_{12}, nicotinamide, nicotinic acid, riboflavin, or thiamine, whereas significant uptake of the first four is detectable in non-resting stages. This particular value of the lymphocyte as a tool for nutritional assessment was suggested by the observation that chronic alcoholics have only half the lymphocyte vitamin B_{12} concentration of normal controls despite serum B_{12} levels almost twice that of the controls. This seemed to indicate that lymphocyte levels may reflect tissue status much more accurately than do serum levels in subjects with abnormalities of circulating transcobalamins.

Disorders of Blood Cell Production

This is an extremely active area, in which results from many new technical advances, such as refinement of culture techniques, are providing a great deal of stimulation. The field is inhibited, by the inadequate supply of purified human erythropoietin.

The aim of some of this research is directed toward correcting the anemia of chronic renal failure, a major problem even in patients who are adequately dialysed. Replacement therapy with exogenous erythropoietin appears promising. Because of the short supply of erythropoietin and of the relative impurity of erythropoietin preparations available, relatively few clinical studies have been reported.

A promising approach to the problem of erythropoietin production might lie in harvesting erythropoietin from large scale cell culture systems. Fetal mouse liver cells grown as a monolayer on the surface of a microcarrier kept in suspension in culture media provide a greatly increased surface area, high cell yield, and good control of important environmental conditions. Erythropoietin production by fetal liver cells has been shown to be stimulated by physiological doses of testosterone and by a lowered pO_2 culture environment. This demonstrated for the first time a direct effect of these on cells capable of producing erythropoietin. Other studies are directed toward understanding the basic processes of normal and abnormal marrow production of blood cells. Investigators have obtained evidence for cell-mediated suppression of bone marrow growth in some aplastic anemia patients. Marrow cultures from approximately 20 percent of patients studied exhibited no erythroid colony formation until T-lymphocytes were removed. After removal, virtually normal colony formation was observed, suggesting that the clinical marrow failure in those patients may be due to direct interaction between circulating cells and the erythroid colony-forming unit in the marrow.

The prototype bone marrow injury resulting in aplastic anemia and leukemia induced by the drug chloramphenicol has been traced to the para-nitroso group of chloramphenicol. This group appears to be the agent which actually induces mutational change. The way is now open for finding the precise mechanism of the drug-induced injury, and perhaps will lead to understanding of the mechanism of development of a number of malignancies.

A very exciting study, in which mouse DNA from a drug-resistant cell line was used to transform bone marrow cells, which were subsequently injected into marrow-depleted mice, has resulted in the first report of a successful gene transplant. The transplanted animals show an increased resistance to the marker drug, thereby raising the hope that patients undergoing cancer therapy could be made capable of tolerating increased doses of therapeutic drugs.

Immunohematology

A number of studies deal with mechanisms of inflammation. In one of these, the ability of derivatives of superoxide dismutase with long plasma half-lives to inhibit induced inflammation in several animal models has been correlated in all cases with an inhibition of the accumulation of inflammatory cells. Thus a role for superoxide may be in the mechanisms by which activated neutrophils might summon additional neutrophils to the site of the inflammation. When normal plasma in vitro is exposed to a source of superoxide production it becomes potentially chemotactic. The generation of this chemotactic activity is inhibited by superoxide dismutase. Development of superoxide dismutase as an anti-inflammatory factor may prove beneficial in the clinical management of inflammation, particularly in chronic or in autoimmune disease states. In other immunologic studies, human erythrocyte surface glycolipids have been found to interact specifically with lectins, and may act as lectin receptors (Goldstein - University of Michigan).

Although it is well-established that lectins interact with glycoproteins of erythrocytes in the binding reaction which leads to agglutination cells A/B/O types, the finding that specific glycolipids have a role in lectin binding will facilitate studies where lectins are used in examining the flux of surface carbohydrates during cellular differentiation and in transport studies.

Iron Chelator Development Program

Requests for Contract Proposals (RIP) were issued, and new contract awards will continue the important effort to develop new, orally-effective iron chelating agents for Cooley's anemia. Several promising agents are awaiting the initiation of toxicologic studies, which are delayed due to the great expense involved in such studies.

KIDNEY DISEASE AND UROLOGY PROGRAM

The Kidney Disease and Urology Program was reorganized in January 1979 when the Artificial Kidney/Chronic Uremia Program was integrated with it. The funds administered by the Kidney Disease and Urology Program during FY 1980, including the support for investigator-initiated research grants, R01s (264), program projects (7), centers (5), contracts (25), Clinical Investigator Awards (14), and Research Career Development Awards (16) exceeded $33.023 million, while the support for National Research Service Awards, both individual (28) and institutional (29) amounted to $2.860 million.

Manpower Development

Two new and 10 renewal institutional National Research Service Awards (T32s) were funded in FY 1980, for a total of 29 active training programs supported by the Kidney Disease and Urology Program, which provide stipends for 89 postdoctoral and 5 predoctoral trainees. Added to the 20 active individual National Research Service Awards (F32) were 8 new awards, for a total of 28 fellowships supported with FY 1980 funds. Three new Research Career Development Awards were made, bringing the total to 16 and the Clinical Investigator Program has been increased to 14, by the addition of 6 new commitments.

Conferences

During FY 1980, the Kidney Disease and Urology (KDU) Program issued awards which provided partial support for the following four international conferences/symposia: (1) Fourth International Symposium on Urolithiasis Research, July 22-26, 1980, in Williamsburg, Virginia; (2) Workshop on Developmental Renal Physiology, October 6-10, 1980, in Philadelphia, Pennsylvania; (3) International Workshop on Ammoniagenesis, June 12-13, 1981, Vouliagmeni, Greece; and (4) Fifth International Workshop on Phosphate, September 24-27, 1981, New York. A conference on Chronic Renal Failure sponsored by KDU was held January 9-11, 1980, and included Symposia on several complications of end-stage renal disease: renal osteodystrophy; neurologic and cardiovascular ones, as well as special problems related to drugs in renal failure and those of immunosuppressive therapy in kidney transplantation. KDU staff is planning actively for two institute sponsored conferences, one on Drugs and Environmental Toxins and another on Neurological Complications of Renal Failure. The former is being co-sponsored with the NINCDS and will be held in late 1981 or early 1982.

RENAL PHYSIOLOGY AND PATHOPHYSIOLOGY PROGRAM

Adult Glomerular Diseases

A multi-center collaborative study of adult glomerular disease was funded recently. This study will attempt to correlate clinical manifestations, course and response to therapy with specific pathologic changes in kidney tissue. Efforts to ascertain any measurable, statistically

significant improvement in mortality and morbidity rates with currently proposed therapeutic agents will be made. The study includes 17 participating institutions, which are to provide approximately 380 patients per year, of whom it is estimated that 275 patients would be willing to enter into the proposed study protocols. The study groups are: (1) adult idiopathic nephrotic syndrome and its response to alternate day prednisone therapy; (2) membranous nephropathy including a randomized prospective trial of cyclophosphamide therapy; (3) a randomized prospective study of plasmapheresis in treatment of rapidly progressive glomerulonephritis; and (4) glomerular disease with non-nephrotic proteinuria.

Immunopathogenesis of Renal Disease

A productive research program involving fundamental studies in both animals and humans on the immunopathogenesis of renal disease is underway at the Children's Hospital Medical Center, Boston, under the leadership of Dr. Fred Rosen. The major focus of this work is upon comprehensive studies of the complement system, including genetic analysis, complement deficiency, alternative biosynthetic pathways and the characterization of C_3 nephritic factor (C_3Nef) IgG. Having a longstanding interest in C_3Nef, this group was the first to recognize that it might be an auto-antibody (IgG) and they have developed, with others, a large body of evidence about its presence in normal subjects. In the outlined future studies, they plan to acquire information about its titer in certain diseases. They anticipate titers will be high and that these findings should add greatly to the understanding of renal disease. Other major research foci have been into studies of the structure of the glomerular capillary; studies of glomerular permeability; about autologous immune complex nephritis; and into human glomerulonephritis. This group is doing ultrastructural studies of autologous immune complex (AIC) nephritis in the rat, which closely resembles human membranous glomerulonephritis. Their findings to date indicate that the nephritis exhibits non-selective proteinuria. Currently they are examining early stages of both active and passive models of AIC nephritis, by perfusing the kidney with autologous anti-BB serum. Since these studies have not been performed previously, the data should be helpful in understanding the pathogenesis of this disease.

In related work in a laboratory of a leading clinical nephrologist at the University of Cincinnati, considerable success has been gained in extending knowledge of human glomerulonephritis (GN), with the extensive clinical, morphological and serological studies that this investigator has directed for the past several years. The work of Dr. Victor E. Pollak and his collaborators has helped characterize and correlate the morphological and clinical aspects of human GN, which has been of great value in establishing criteria for its treatment.

Developmental Renal Physiology

The object of a project at the Kansas University Medical Center is to characterize the passive movement of fluid and solute across the peritubular

membrane of proximal tubules in the developing kidney. Its ultimate goal is determination of whether or not the permeability of the peritubular membrane changes during development. The studies conducted have been successful in identifying and accurately localizing specific segments of the proximal tubule. Physiological data accumulated thus far indicate that, qualitatively, the response of developing tubules to ouabain is similar to that of the adult tubule and, that quantitatively the degree of swelling is similar also. For instance, an increase in cell size approximately 40 percent was noted 60 minutes following the addition of 10^{-4}M ouabain. Studies such as these should provide some clarification of the changes that take place in fluid transport across the renal tubule cells as the kidney matures.

Kidney Prostaglandin Synthesis in Tissue Culture

The kidney is an important source of prostaglandin production. However, considerable controversy remains about the role which the renal prostaglandins may play in modulating renal function in normal and in diseased states. Due to the difficulty in ascribing renal prostaglandin production to a specific nephron segment or to a specific region of the kidney in whole animal experiments, a group at the University of Texas, San Antonio, is studying prostaglandin production in glomerular cells grown in tissue culture. A type 2 cell begins to appear about 2-3 weeks after in vitro culture. Although not absolutely proven, this cell type appears to resemble a mesangial cell, as based on its growth characteristics, and upon its light and electron microscopic appearance. To determine whether or not these cells synthesize prostaglandin, the prostaglandin precursor, ^{14}C arachidonic acid was added to the medium containing type 2 cells. The findings to date indicate that these cells incorporate arachidonic acid and that arachidonic acid is metabolized to prostaglandins. The ability of isolated cells to metabolize ^{14}C arachidonic acid to prostaglandins was inhibited by indomethacin and meclofenemate.

Renin and Hemodynamics

Potassium (K) depletion is known to cause changes in hemodynamics and in plasma renin activity (PRA). In investigations directed by Dr. Stuart L. Linas at the University of Colorado School of Medicine, studies of the relative roles of K, angiotensin, and prostaglandins in controlling hemodynamics during dietary K-deficiency are underway. The mechanism of hyperreninemia observed during chronic K-deficiency is being studied also. Results to date indicate that 14 days of dietary K-deficiency causes marked changes in both systemic and renal hemodynamics. Moreover, PRA was observed to be markedly increased two to three fold in K-deficient animals. To eliminate many factors which could alter renin release, this investigator utilized the isolated perfused kidney to elucidate the mechanism of renin release in K-deficiency. In those kidneys perfused at 120 mm Hg, Dr. Linas found increased renin levels. According to this investigator's interpretation of his results, the increase in renin levels is associated with a decrease in distal nephron fluid delivery

and an increase in renal baroreceptor tone. The former is as estimated by urine flow and the latter by renal vascular resistence. Consequently, renin release could be caused by stimulation of either the macula densa or the baroreceptor, or by a direct effect of K-deficiency. He was able to demonstrate that the effect is not a result of acute hypokalemia since perfusing normal kidneys with hypokalemic (2.5 vs 5 mm K) solutions did not increase renin levels. The results of these investigations appear to eliminate the involvement of the macula densa mechanism. These results suggest that the increase in renin in K-deficiency is mediated by the baroreceptor or that it is the direct effect of K-deficiency.

Neurohypophysial Regulation of Water Excretion

A long-time grantee of the NIAMDD, working at Columbia College of Physicians and Surgeons, continues to be productive in the design and synthesis of analogues of oxytocin and vasopressin. His aim is to develop potent competitive antagonists to: (1) the antidiuretic response; (2) the vasopressor response; and (3) the oxytocic response. If such antagonists can be successfully developed, he plans to study the functions of the endogenous neurohypophysial hormones in cardiovascular regulation and in the initiation of labor. If a safe and effective antagonist of the antidiuretic response can be developed, its most promising experimental use would be in clinical conditions in which elevated rates of antidiuretic hormone (ADH) release may contribute to water retention and dilutional hyponatremia. In addition to the obvious, "syndrome of inappropriate ADH secretion", such conditions as the dilutional hyponatremia associated with severe congestive heart failure and the blunted water diuresis in adrenocortical failure should be identified.

Kidney and Urinary Tract Stones

--Treatment: Research in progress supported by the NIAMDD is aimed at defining more specific causes for renal stones. For example, recent studies have demonstrated a statistical correlation between calcium oxalate stone formation and excretion of large quantities of uric acid in the urine of patients, a disorder distinguishable from other types of calcium stone formation. The mechanism of this type of stone formation is not known. A facilitating role is suggested for the hyperuricosuria crystals of the sodium salt of uric acid and those of calcium oxalate. The results of a study by Dr. Charles Y. Pak and associates of the University of Texas Southwestern Medical School lends support to the theory previously reported by another NIAMDD grantee, Dr. Frederic L. Coe of the Michael Reece Hospital and the University of Chicago. This theory proposes that a purine-rich diet promotes calcium-containing kidney stone formation by increasing renal excretion of uric acid and by supersaturating urine with monosodium urate, thus favoring crystallization of calcium salts, directly or indirectly, by removing inhibitors of crystal growth. Furthermore, the investigators have found that allopurinol therapy decreases urinary uric acid and saturation with monosodium

urate, thus inhibiting calcium-containing stone formation. This ultimately yields significant clinical benefit by diminishing the number of stones formed.

--Etiology and Diagnosis: In other research by Dr. Pak and his associates, evidence suggests that abnormal handling of calcium by the small intestine or by the kidney may be responsible for urinary excretion of excess calcium and the resultant increased kidney stone formation. This study, under the direction of Dr. John Fortran, was undertaken to localize precisely the intestinal site where excessive calcium absorption occurs in patients with kidney stones. He reported evidence to indicate that such a condition in patients with defective intestinal absorption and excess urinary calcium predisposing to kidney stones may be due to the enhancement of calcium transport from the lumen to the blood vessels in the jejunum. It was suggested by this group that this process may be mediated by high levels of a Vitamin D analog.

--Epidemiology of Urolithiasis: The work of another Institute grantee, Dr. Paul Stolley at the University of Pennsylvania, complements the data being compiled by Dr. Pak's group. Dr. Stolley is conducting an epidemiologic study to identify a population of persons with hypercalciuria (excess calcium in the urine) in the Philadelphia area. Data gathered in this study will provide the basis for prospective research in which the investigator plans to compare the incidence of urolithiasis between a hypercalciuric population and a group of people with normal levels of calcium in the urine. Dr. Stolley hopes to define possible risk factors, such as: obesity, occupation, use of medications, and urinary tract infections. His work offers the prospect of significantly enlarging present knowledge of the role of calcium metabolism in the etiology of urolithiasis as well as elucidating the importance of genetic and environmental influences upon development of the renal stone disease.

--Transport of Ureteral Calculi: Renal colic (pain caused by ureteral calculi lodged in and/or passing down in the ureter) is one of the most common medical/surgical emergencies and causes major suffering, as well as potential permanent damage to the kidney and/or ureter. At present, there is no rational medical management that will facilitate transport of calculi down the ureter. This possibility is being studied by Dr. Michael E. Mayo at the University of Washington School of Medicine. As part of his investigations, Dr. Mayo is studying the effects of various rates of urine flow on ureteral activity and the effects of numerous drugs on activity of the normal ureter at various flow rates. The researcher is attempting to determine which factors improve or hinder the movement of kidney stones down the ureter by a series of experiments. He reports that one of the most interesting findings so far is the marked inhibitory effect of glucagon on the process of stone movement in the ureter. This agent, a physiologic one, which is already in common clinical use may prove to have an important clinical application in treatment of ureteral calculi.

Vesicoureteral Reflux

The management of children with vesicoureteral reflux remains controversial. Therefore a multi-center prospective study which was recently funded through the Urology (Including Urolithiasis) Program should help to define more clearly the optimal treatment for children with this problem. A proportion of the children with this malady show retardation of renal growth, progressive renal scarring and, in a few cases, develop loss of renal function. It is unclear whether reflux alone, or reflux and infection, or reflux with obstruction is responsible for these changes; and whether surgical correction of the reflux prevents or arrests any of these changes. This prospective, randomized study, involving the collaboration of 15 institutions caring for pediatric patients, will determine whether children under the age of nine with Grade III* vesicoureteral reflux are best treated by prophylactic antibiotic therapy or by surgical correction using ureteral reimplantation. Medical treatment will include anti-bacterial therapy, using small doses of drugs for as long as the reflux persists and for six months thereafter. Followup studies will be at six months, two years, and five years in the operative group; and at six months, two years, three and a half years, and five years in the medically-treated group. Two episodes of urinary tract infection while under antibacterial treatment will constitute a failure of medical treatment. It is estimated that 400 children will need to be studied to detect a significant difference.

CHRONIC RENAL DISEASE PROGRAM

Developments in Kidney Transplantation and Artificial Kidneys

The NIAMDD is one of the several institutes of the National Institutes of Health which supports research into transplantation. This includes fundamental immunology, immunosuppressive treatment, techniques of surgery, and organ preservation. Transplantation is an effective means for treatment of patients with end-stage renal disease when well-matched related donor kidneys are available, but when only cadaver kidneys are obtained it is less effective. The requirement for long-term immunosuppressive therapy because of the potential for rejection and the high incidence of graft failure has resulted in less consistent results than those by treatment with dialysis, in many instances. Overcoming the problem of rejection of grafted kidneys continues to be one of the most serious obstacles to a predictable outcome in the use of transplantation, although there has been progress in this area in recent years. In general, patients who receive well-matched organs from closely related donors have the best chance of success among recipients of transplants and of a consequent return to a more normal exsistence.

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*Grade III vesicoureteral reflux is defined as uteteral and pelvicalyceal filling, clubbing of the calyces with minor to moderate pelvic dilatation, and slight ureteral tortuosity.

The main technique used in treatment of chronic renal failure for more than fifteen years has been maintenance dialysis (primarily hemodialysis, and also peritoneal dialysis). In 1979 dialysis was provided for about 46,000 patients under the Medicare End-Stage Renal Disease (ESRD) Program while transplantation was provided for about 4,000 persons in the U.S.A. in 1979 under Medicare. When compatible donors are available, transplantation is a useful alternative.

Many complications arise during the course of treatment of patients by maintenance dialysis. Chronic renal failure and its accompanying "uremia" is a multi-organ system disorder. The problems arise secondary to dialysis treatment and, also regardless of treatment, in relation to the uremic state. Examples of these complications include: neuropathy, primarily impaired sensation and/or impaired muscle strength in limbs; renal osteodystrophy; increased susceptibility to infection, central nervous system symptoms, cardiovascular complications, anemia, and sexual dysfunction. In addition, the well-being of patients on hemodialysis is impaired by occasional muscle cramps, hypotension and post-dialysis fatigue, especially when large amounts of fluids have been removed during dialysis treatments, which usually are given three times each week. The mortality rate among maintenance dialysis patients is about nine percent a year regardless of sex or age. This figure must be viewed in contrast with certain 100 percent mortality for patients with non-functional kidneys if dialysis or transplantation were not available for treatment.

Blood Transfusions Favorably Affect Survival of Kidney Grafts

Because of the limited supply of kidney transplants from compatible closely related, living donors, the majority of transplant patients with end-stage renal disease receive cadaver kidney grafts. The overall rate of functional graft survival remains substantially below that for transplants from living donors. In an effort to improve graft survival, NIAMDD grantees Dr. Oscar Salvatierra, Jr., Dr. Paul I. Terasaki, and respective associates at the University of California Medical Centers in San Francisco and Los Angeles have studied the effects of various pre-transplant immunologic factors, including blood transfusions, tissue compatibility antigens, and antibodies accelerating graft rejection in cadaver kidney recipients. Graft survival data were analyzed at one transplant center in San Francisco, which also used data from participating centers in Los Angeles. Results show that success of cadaver kidney transplants increase in proportion to the number of blood transfusions the patient had received previously while on maintenance dialysis. As the result of this work and similar studies in Western Europe, it is now recommended that candidates for eventual cadaver kidney transplants receive multiple blood transfusions during their transplant-anticipating period on maintenance dialysis.

Use of 24,25 - Dihydroxy Vitamin D_3 in Dialysis Osteomalacia

Use of a mixture of 24,25 dihydroxy vitamin D_3 (24,25) and 1,25 dihydroxy vitamin D_3 (1,25) was found to be effective in dialysis patients with

refractory osteomalacia. This interesting finding was made by Dr. Jack Coburn and associates of the University of Los Angeles. Treatment of these patients with 24,25 alone, or with 1,25 alone produced no clinical improvement. The patients previously had received either vitamin D_2 or dihydrotachisterol (DHT) without beneficial response. The investigators also found that combinations of 24,25 and DHT were effective in increasing bone mineralization. Following the combined therapy, patients exhibited lower serum calcium and acid phosphatase, with bone biopsies demonstrating a lowering of osteioid volume.

Preservation of Renal Function in an Animal Model of Experimental Glomerulonephritis

A group of investigators at the University of Colorado have demonstrated that phosphorus restriction in the diet retards functional deterioration in experimental immunologic renal disease. Dr. Alfrey and his colleagues also have shown reduced histologic damage in the rat model in the restricted phosphorus group (group B). Other observations were that the group B animals survived longer, had lower serum creatinine levels, and lower total kidney calcium. The investigators note that the mechanisms for this protective effect are unknown. The phenomenon of diminished symptoms of renal disease in patients on restricted protein (with implicit restricted phosphate) diet has been observed earlier by other grantees, including Dr. Neal Bricker who enunciated the "trade-off" hypothesis and by Dr. Mackenzie Walser and his associates of the Johns Hopkins University. Other studies in both animal models and in patients have contributed to an understanding of the disease process.

Treatment of Intractable Uremia Pericardial Effusion

Pericardial effusion complicating dialysis is considered intractable when it does not respond to conservative management of uremia, improved dialysis regimens, or systemic therapy with anti-inflammatory drugs. As an alternative to the surgical excision (stripping) of the pericardium (a sac-like structure enveloping the heart) indicated in such cases, Institute grantees Dr. Richard L. Simmons and Dr. John S. Najarian of the University of Minnesota have instilled locally a nonabsorbable steroid through an indwelling pericardial drainage catheter. This procedure, which has been used in 45 patients, has reduced morbidity, mortality, and length of hospital stay of uremic patients with intractable pericardial effusion at three different medical centers during the past four and a half years. The researchers concluded that the effectiveness of combined percutaneous catheter drainage and local nonabsorbable steroid instillation make this technique superior to surgery in management of pericardial effusion in uremic patients.

Comparative Study of Androgen Therapy for Anemia in Hemodialysis Patients is Completed

The relative effectiveness of four drugs for treatment of the anemia in uremic hemodialysis patients was compared by Dr. Martin Neff and his

co-investigators of the Mount Sinai School of Medicine, New York. A random cross-over study design, including control periods interspersed between drug treatment periods was used. Each patient received up to three drugs in the course of the study.

According to a preliminary data analysis, favorable response of patients to the injectable drugs, Decodurabolin and Delatestryl, was statistically greater than that for Halotestin and Anadrol. Female patients were not given Delatestryl. Mean hematocrit increases compared to the control period for the injectable drugs were in the range of 5-6 percent, with maximum hematocrits in range of 29.5-31.5. These results provide guidance for the clinical management of dialysis patients with severe anemia.

Hollow-Fiber Ultrafilters for Removal of Bacteria and Toxic Products

Continual purification of the blood of uremic patients undergoing peritoneal dialysis or the new extracorporeal hemofiltration process requires large quantities of expensive solutions free of bacteria and their fever-producing contaminating products (pyrogens). In an effort to reduce the cost of such solutions, NIAMDD contractor Dr. Lee W. Henderson of the Veterans Administration Hospital and the University of California in San Diego has tested hollow-fiber ultrafilters made of highly porous artificial membranes for their capacity to remove bacteria and pyrogens from typical dialysis fluid. So far the results of using these ultrafilters in the sterilization process have been successful in the cases of several patients in whom solutions sterilized and purified by ultrafiltration are being infused during routine maintenance hemofiltration treatment for uremia. This low-pressure filtration process may be accomplished in one step, and is adaptable to solutions containing pre-treated municipal tap water. If quality control can be maintained the clinical application of these solutions could reduce significantly the cost of peritoneal dialysis or hemofiltration treatment.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATIONS

The Office of Program Analysis and Scientific Communications (OPASC) is responsible for developing and executing a comprehensive program for the planning, analysis, evaluation and reporting of the Institute's activities and policies, as well as for advising the Institute Director and his immediate staff concerning the implications of pending health legislation and of scientific and policy-relevant reports.

Planning

A major responsibility of the Office is preparation of the Institute's Annual Research Plan, which is preceded by the Institute Director's review of each extramural cluster's research accomplishments, current activities, and projections concerning scientific opportunities and budgetary needs. During fiscal year 1980, OPASC staff prepared and distributed guidance concerning the Director's review sessions; assisted NIAMDD program staff in preparing for their oral and written presentations; developed the Institute's Annual Research Plan following analysis and synthesis of programmatic data; and prepared briefing materials for use by Institute management during the NIH Director's annual review of NIAMDD planning activities. The Office also supplied input to the Public Health Service Program Plan, the NIH Health Services Research Plan, and the Report of the Department of Health and Human Services Steering Committee for the Development of a Health Research Strategy. In addition, the Office developed materials for briefing the Institute's National Advisory Council on NIAMDD program planning activities.

Program Analysis

The past year witnessed a continued increase in the number of program analysis requests received and responded to by OPASC staff. These requests typically require a review of the Institute's entire research portfolio to determine the research grants, training grants, center grants, intramural projects and other activities related to the disease, scientific discipline or other subject matter of inquiry; calculation of the funds expended or projected to be expended in the relevant areas; and preparation of explanatory narrative material. Following are examples of program analyses performed by OPASC staff during fiscal year 1980:

- Rehabilitation Research
- Population Research
- Alcohol and Alcohol Abuse
- Toxicology
- Drug Abuse Research
- Preservation of Germplasm Resources

OPASC also supplied substantial analytical input into a number of documents, including the following:

- o NIH Annual Report on International Activities
- o Prevention '79
- o Interagency Technical Committee (IATC) Report on Federal Programs in Heart, Lung, and Blood Diseases and Blood Resources

Reports

During fiscal year 1980, OPASC staff prepared ad hoc reports on hypoglycemia and on NIAMDD current and planned activities in kidney disease, urology and nephrology. Both of these reports were requested by the Senate during fiscal year 1980 Appropriations Hearings.

The Office also prepared the Third Annual Report of the NIAMDD Director on the Arthritis Program for transmittal to Congress and the President. This report is one of six statutory reports which the Institute prepares annually and for which the OPASC serves as the central point for review, clearance, and official transmittal to the Secretary and/or the Congress. Clearance of a variety of ad hoc and special Institute reports is also a continuing responsibility of OPASC staff.

To provide a central perspective on Institute activities, the OPASC has recently made plans for a new initiative -- an annual report of the NIAMDD Director. This report will contain retrospective, current, and prospective data on programmatic activities and resources, and thus, will serve as a highly useful compendium of information for Institute staff, the biomedical research community, voluntary health organizations, the Congress, and other individuals and groups having a particular interest in NIAMDD. It is expected that the first NIAMDD Director's Report will be published in 1981.

Evaluation

The OPASC prepares the Institute's Annual Evaluation Plan and collaborates with extramural clusters in originating, initiating, and monitoring individual NIAMDD evaluation projects.

As directed by the Senate, the Institute completed two major evaluation studies during fiscal year 1980: An Evaluation of Research Needs in Endocrinology and Metabolic Diseases and Analysis of Priorities and Needs for Research in Dermatology. An evaluation of the complications of dialysis is nearing completion and a study of the Institute's Hematology Program will be concluded during 1981.

In the upcoming months, the Institute plans to initiate several new evaluation studies involving the Musculoskeletal Diseases Program, various NIAMDD Center Grant Programs, and the National Diabetes Information Clearinghouse.

Medical Applications of Research

As NIAMDD's official point of liaison with the NIH Office of Medical Applications of Research (OMAR), the OPASC is responsible for recommending to the Institute appropriate areas for consensus development conferences; reporting to the NIH Director on emerging technologies identified by scientific staff as having the potential for impacting on medical practice; and representing the Institute at OMAR meetings and conferences.

During fiscal year 1980, OPASC staff also responded to frequent requests from OMAR to review the state-of-the-science with respect to various drugs, devices, and techniques whose eligibility for coverage through Medicare was under active consideration by the Health Care Financing Administration. Medicare coverage issues addressed by OPASC included the following:

- Charcoal Filter Detoxification
- Ultraviolet Light Treatment in Uremic Pruritis
- Medical Interventions Following Acute Episodes of Serum Hepatitis
- Uroflowmetric Evaluation
- Use of Cryoprecipitate Techniques in Extraction of Renal Calculi
- Blood Self-Administered by Home Dialysis Patients
- Gastric Bypass Surgery
- Electromagnetic Treatment of Fractures
- Liver Transplantation

Legislation

For NIAMDD, fiscal year 1980 was particularly active legislatively, with a variety of bills proposed in diabetes, arthritis, and digestive diseases. In addition, Senator Kennedy and Congressman Waxman introduced bills (the Health Sciences Promotion Act and the Health Research Act, respectively) that would extensively revise the Public Health Service Act, resulting in major changes in the statutory authorities and requirements of NIAMDD and other NIH components.

OPASC staff performed detailed analyses of pending legislation pertinent to the Institute; attended Congressional hearings; prepared legislative briefing materials for use by Institute management and by the NIAMDD National Advisory Council; developed NIAMDD legislative proposals; and prepared a number of bill reports outlining the Institute's position with respect to legislation relevant to NIAMDD.

Review

Throughout the year, OPASC staff have supplied critical comments on a variety of documents referred to the Institute for review. These included:

- Regulations Governing the National Institute of Handicapped Research
- Regulations on Federal Paperwork Reduction
- Women and Health, U.S.A., 1980
- OTA Commissioned Study on the Usefulness and Implications of Cost-Effectiveness Analysis and Cost-Benefit Analysis in Health Care Decision-Making
- Pan American Health Organization Documents on Diarrheal Disease Control, Noncommunicable Diseases Control, and Malnutrition and Deficiency-Induced Mental Retardation.
- Surgeon General's Report on Disease Prevention and Health Promotion
- World Health Organization Document: Development and Coordination of Biomedical and Health Services Research

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS
AND SCIENTIFIC COMMUNICATIONS

SCIENTIFIC COMMUNICATIONS SECTION

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National Digestive Diseases Education and Information Clearinghouse

The Scientific Communications Section had the responsibility for general planning of communication activities concerned with the various scientific disciplines that are charged to NIAMDD. Activities were grouped into four main categories:

1. Current-awareness literature services;
2. Retrospective literature studies;
3. Conferences and workshops;
4. Other aspects of communication, such as thesaurus development, production of motion pictures, etc.

Current-awareness literature services were provided by the Institute in several specialized areas and included four publications with hierarchically arranged listings of literature citations. These publications were: ENDOCRINOLOGY INDEX, DIABETES LITERATURE INDEX, INDEX OF DERMATOLOGY, and KIDNEY DISEASES AND NEPHROLOGY INDEX. The publications contained citations to current worldwide literature that has been included by the National Library of Medicine in its Medical Literature Analysis and Retrieval System (MEDLARS) and also citations gleaned from other sources of biomedical literature.

To produce these structured, hierarchically-arranged INDEXES that functioned as indepth current-awareness tools for comprehensive review of particular subjects, cooperation with the National Library of Medicine, and in one case with the University of Minnesota and the University of Rochester, was necessary. The standardized terminology used in MEDLARS made these publications valuable for retrospective literature searching to those interested in these specialized areas.

When the development of these publications was undertaken in the late 1960's and early 1970's, it was both difficult and time-consuming to locate relevant literature in the field. With this lack of resources, in order to move the field along, and as part of the support of research, NIAMDD undertook publication of these INDEXES. Today there are more and different resources available for finding citations and abstracts of the literature in these fields than there were a number of years ago, and there is no longer the void that originally prompted the development of these publications.

With limited financial resources available to support research, and in view of the availability of other literature sources, the Institute discontinued the INDEXES. The last issues were: ENDOCRINOLOGY INDEX, Volume 12, No. 6; DIABETES LITERATURE INDEX, Cumulative Volume 14; INDEX OF DERMATOLOGY, Volume 11, No. 12; and KIDNEY DISEASE AND NEPHROLOGY INDEX, Volume 5, No. 6.

Conferences, symposia, and workshops were under the auspices of the Section. This year, six conferences and workshops were sponsored by NIAMDD that involved the Section. These included the Arthritis Epidemiology Conference, Chronic Renal Disease Conference, Directions for the Proposed National Digestive Diseases Education and Information Clearinghouse, Endoscopy, What is Its Role in Upper Gastrointestinal Bleeding?, Intact Human Cell Lives in the Study of Inborn Metabolic Diseases, and the Workshop on Regulation of Hepatic Cholesterol and Bile Acid Metabolism.

Proceedings and conference summaries included the Twelfth Annual Contractors' Conference Proceedings, Fourth International Conference on Bone Measurement Proceedings, and the Methodologies for Human Population Studies in Nutrition Related to Health Proceedings.

National Library of Medicine's on-line MEDLARS was available to NIAMDD staff through NIAMDD's call number. The Scientific Communications Section was responsible for coordinating this use and served as a reference source for all of the NIAMDD staff (Extramural, Intramural, and Administrative).

Numerous other activities that bore on scientist-to-scientist communication affecting the Institute were dealt with frequently. One of the most important new efforts that the Section helped with its initial efforts was the new National Digestive Diseases Education and Information Clearinghouse. This new endeavor was undertaken by the Institute as the result of the recommendations made by the National Commission on Digestive Diseases in their "Report to the Congress." In response to the Commission's recommendations, the Clearinghouse will consist primarily of a planned outreach educational and informational program with a clear, realistic focus and appropriate methods of evaluation.

Project Title: DIABETES LITERATURE INDEX

Contract Number: N01-AM-7-0663

Contractor: University of Minnesota

Project Officer: Mrs. Billie B. Mackey

Man Years:

Total	.30
Professional	.10
Other	.20

Project Description:

The DIABETES LITERATURE INDEX was a current-awareness publication produced monthly with a yearly cumulative issue. This bibliography of diabetes-related literature was brought about through the cooperative efforts of NIAMDD, the National Library of Medicine, the American Diabetes Association and three universities, (University of Minnesota, University of Rochester, and Case Western Reserve University), as well as many individual scientists.

The National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS) referencing all of the biomedical literature citations to both journals and monographs, was used to retrieve all diabetes-related bibliographic citations monthly. Out of this retrieval, approximately one third of the citations of interest to diabetologists and others working in the diabetes field were carefully selected and culled from the peripheral literature.

Three formats were included in the publication, a hierarchical subject index, an abbreviated keyword in title index, and an author index that included index terms with the citations.

When the publication DIABETES LITERATURE INDEX was initiated in 1966, it was both difficult and time consuming to locate relevant literature in the field. With this lack of resources, in order to move the field along, and as part of the support of research, NIAMDD undertook publication of DIABETES LITERATURE INDEX. Today there are more and different resources available for finding citations and abstracts of the literature in the field than there were a number of years ago, and there is no longer the void that originally prompted the development of DIABETES LITERATURE INDEX.

In view of the limited resources of the Institute for research programs and the availability today of other literature sources, the Institute discontinued the DIABETES LITERATURE INDEX activities. The last issue was Volume 14, Cumulative Issue, 1979.

DIABETES LITERATURE INDEX was published by the Scientific Communications Section, NIAMDD and available free to grantees or contractors with interests in diabetes, government agencies with responsibilities in the area, and to medical school libraries, and was for sale by the Superintendent of Documents, U.S. Government Printing Office for a fee.

Project Title: ENDOCRINOLOGY INDEX

Professional Personnel: Mrs. Billie B. Mackey

Man Years:		
	Total	.50
	Professional	.15
	Other	.35

Project Description:

The ENDOCRINOLOGY INDEX covering approximately 4,000 citations of the world literature in journals and monographs of interest to those in the field of endocrinology, is a bimonthly current-awareness journal published by the Scientific Communications Section with the aid of the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS). The format of the publication consists of citations grouped in eight categories (pituitary, thyroid, etc.), and each category has a preconceived hierarchical organization of subject headings. Index terms, assigned by the indexers, after pursuing the original article, appear in the author section, and author and subject indices appear in each issue.

When the publication ENDOCRINOLOGY INDEX was initiated in 1968, it was both difficult and time consuming to locate relevant literature in the field. With this lack of resources, in order to move the field along, and as part of the support of research, NIAMDD undertook publication of ENDOCRINOLOGY INDEX. Today there are more resources available for finding citations and abstracts of the literature in the field than there were a number of years ago, and there is no longer the void that originally prompted the development of ENDOCRINOLOGY INDEX.

In view of the limited resources of the Institute for research programs and the availability today of other literature sources, the Institute decided to discontinue the ENDOCRINOLOGY INDEX. The last issue was Volume 12, Issue 6.

ENDOCRINOLOGY INDEX was available free to National Institutes of Health grantees and contractors with interests in the field, related government agencies, and medical school libraries, and was for sale by the Superintendent of Documents, Government Printing Office for a fee.

Project Title: INDEX OF DERMATOLOGY

Professional Personnel: Mrs. Elsie C. Yuen

Man Years:

Total	.25
Professional	.15
Other	.10

Project Description:

To support the NIAMDD Dermatology Program, and respond to an increased interest and demand for up-to-date clinical studies and research information in dermatology, NIAMDD has been publishing the INDEX OF DERMATOLOGY, a monthly recurring bibliography, since April 1972. However, as financial support for research in dermatology becomes more limited, and resources not available earlier are found to meet this need, it has been necessary to discontinue publication of the INDEX with Volume 12, Number 12 (December 1979 issue).

About 1,300 dermatology-relevant citations were computer-selected each month from MEDLARS and grouped under specific subject headings, according to their clinical or research orientation. The retrieval was monitored and edited to assure that relevant citations were not omitted, and the formulation was updated annually to keep abreast of changes in the MEDLARS controlled vocabulary.

The INDEX OF DERMATOLOGY has been available free to qualified investigators with NIH grants or contracts, to related government agencies, and to medical school libraries. It was also for sale by the Superintendent of Documents, Government Printing Office, at nominal cost.

Project Title: KIDNEY DISEASE AND NEPHROLOGY INDEX

Professional Personnel: Mrs. Elsie C. Yuen

Man Years:

Total	.20
Professional	.10
Other	.10

Project Description:

To support research in the field, NIAMDD has been publishing since 1975 the KIDNEY DISEASE AND NEPHROLOGY INDEX, a bimonthly recurring bibliography containing the most recent literature citations. Together with its predecessor the ARTIFICIAL KIDNEY BIBLIOGRAPHY, the publication has been a valuable research tool for investigators in the field of nephrology. However, as research support funds become more limited, and resources not available earlier are found to meet this need, it has been necessary to discontinue publication of the INDEX with Volume 5, Number 6 (November-December 1979 issue).

Approximately 2,250 citations were computer-selected every two months from the current MEDLARS data base and placed under appropriate subject headings in an organized hierarchical arrangement. The retrieval was monitored and edited by the staff before publication. The search formulations were updated annually to reflect changes in the controlled vocabulary. Technical reports from the Chronic Renal Disease Program deposited with the National Technical Information Service (NTIS) were also listed by title, twice a year, in the INDEX.

The KIDNEY DISEASE AND NEPHROLOGY INDEX has been available free to qualified NIH agencies, and to medical school libraries. It was also placed on sale by subscription from the Government Printing Office.

Project Title: ARTHRITIS EPIDEMIOLOGY CONFERENCE

Professional Personnel: Mrs. Reva Laurence
Mrs. Billie B. Mackey
Mrs. Elsie C. Yuen

Project Description:

This three-day conference on the epidemiology of arthritis, held September 24-26, 1980 in Bethesda, Maryland, brought together an interdisciplinary group of rheumatologists and epidemiologists who focused their attention on the investigation of the rheumatic diseases through the discipline of epidemiology. They reviewed the current state of epidemiologic knowledge of the rheumatic diseases and defined gaps in that knowledge. To move the field forward, they identified research opportunities, proposed research strategies, and determined required resources.

The proceedings of this "state of the art" conference will be published along with a comprehensive review of the arthritis-epidemiology literature in the form of an annotated bibliography.

Project Title: CHRONIC RENAL DISEASE CONFERENCE

Professional Personnel: Dr. Nancy B. Cummings
Dr. Antonia Novello
Mrs. Billie B. Mackey
Mrs. Elsie C. Yuen

Man Years:

Total	.10
Professional	.05
Other	.05

Project Description:

The Chronic Renal Disease Conference was held January 9-11, 1980 in Bethesda, Maryland. Approximately 350 individuals interested in all aspects of chronic renal disease were in attendance.

The formal sessions were primarily "state-of-the-art" presentations and covered topics such as renal osteodystrophy, vitamin D analogs and parathormone; transplantation and immunosuppression; drugs and renal failure and acute problems during hemodialysis; and the neurologic and cardiovascular complications of renal failure.

Poster sessions, as an informal communication tool, included two sessions scheduled over two days to cover 39 presentations, and were combined with the late afternoon social hour after the formal sessions which drew a sizeable number of viewers. Poster sessions covered the topics of (1) Transplantation and Pathophysiology and (2) Dialysis.

Attendance at the conference had Continuing Medical Education approval for 14 hours.

Project Title: DIRECTIONS FOR THE PROPOSED NATIONAL DIGESTIVE DISEASES EDUCATION AND INFORMATION CLEARINGHOUSE

Professional Personnel: Dr. Kirt J. Vener
Dr. Harold P. Roth
Mrs. Billie B. Mackey

Man Years:

Total	.1
Professional	.06
Other	.04

Project Description:

The need for a National Digestive Diseases Education and Information Clearinghouse was described in the report of the National Commission on Digestive Diseases. As a planning measure, NIAMDD conducted an open meeting to learn of the interest, goals and plans as well as current and past activities of those organizations involved in patient and public education in this important area.

The meeting was held on January 21, 1980 at NIH with the objective of providing background information for designing an implementation plan for the National Digestive Diseases Education and Information Clearinghouse. Congressman Tim Lee Carter, M.D., discussed bills he had introduced into Congress to establish the Clearinghouse. Other speakers included Federal agency representatives who discussed the origin and objectives of their programs, the task assigned, funding levels, and their relationship with voluntary health groups. The representatives from the voluntary health groups discussed the history and mission of their organizations and gave a description of how their organizations would interact with a national information clearinghouse funded by NIAMDD. They identified tasks having high priority for the Clearinghouse.

On the second day, January 22, 1980, a consultant panel met to discuss the information presented and the draft recommendations.

Project Title: ENDOSCOPY- WHAT IS ITS ROLE IN UPPER GASTROINTESTINAL BLEEDING?

Professional Personnel: Dr. Harold P. Roth
Dr. Kirt J. Vener
Mrs. Billie B. Mackey
Mrs. Elizabeth Singer

Project Description:

The Consensus Development Conference on Endoscopy—What is Its Role in Upper Gastrointestinal Bleeding? was held in Bethesda, Maryland on August 20-22, 1980.

This consensus development meeting was convened to address issues that have arisen over the use of endoscopy in upper gastrointestinal bleeding. The goal of the conference was to discuss the relevant views of practicing physicians and experts in gastroenterology, liver diseases, endoscopy, radiology, and biostatistics, so that general agreement could be reached on the circumstances appropriate for the use of endoscopy.

Presentations addressed the benefits and risks of upper gastrointestinal bleeding, alternative methods of diagnosis and management of upper gastrointestinal bleeding, and indications and contraindications for the use of endoscopy.

Project Title: INTACT HUMAN CELL LINES IN STUDY OF INBORN METABOLIC DISEASES

Professional Personnel: Dr. Robert Katz
Mrs. Billie B. Mackey

Project Description:

This 1½-day workshop held June 12-13, 1980 at Bethesda, Maryland was planned because of an identified need for interaction among scientists in many disciplines who were working on metabolic research in intact cells. A major objective was to aid communication between active researchers in different areas of intact-cell research and to emphasize the use of intact human cell lines in studying inborn errors of metabolism.

The workshop was divided into two sessions, and covered the role of intact cells in purine and pyrimidine metabolism, and other selected topics. Biochemical and genetic aspects were emphasized with a round table discussion during each session. These discussions covered the present status and expected developments in the field, as well as research topics of mutual interest. The workshop identified research areas that need emphasis and support.

Publication of the proceedings including the round table discussions will carry the workshop's message to the scientific community at large. The publication is also planned to initiate a NIAMDD monograph series in metabolism and metabolic diseases.

Project Title: WORKSHOP ON REGULATION OF HEPATIC CHOLESTEROL AND BILE ACID METABOLISM

Professional Personnel: Dr. Sarah C. Kalser
Mrs. Billie B. Mackey

Project Description:

The purpose of this workshop, held October 19-21, 1979 was to bring togther active researchers in the bile acid-cholesterol-lipid field to discuss the current state-of-the-art and to identify future research needs. Approximately 70 established scientists and young investigators participated.

The sessions addressed the topics of lipid uptake by the liver including data presented on chylomicron remnants, chylomicrons and very low density lipoproteins, low density lipoproteins, and high density lipoproteins; regulation of triglyceride and phospholipid synthesis; regulation of hepatic cholesterol synthesis; regulation of bile acid synthesis as affected by negative feedback, compartmentalization, diurnal variation and sources of cholesterol for bile acid synthesis; and sources of bile lipids and the role of bile acid in regulating bile secretion.

A summary of the workshop will be published in the Journal of Lipid Research.

Project Title: PROCEEDINGS OF THE FOURTH INTERNATIONAL CONFERENCE ON BONE MEASUREMENT

Professional Personnel: Mrs. Elsie C. Yuen
Mrs. Billie B. Mackey

Man Years:

Total	.95
Professional	.65
Other	.30

Project Description:

This publication documents the proceedings of the Fourth International Conference on Bone Measurement held at the University of Toronto, Ontario, Canada from June 1-3, 1978. The sixth meeting of its kind held since 1959, this conference has provided a valuable forum for investigators from diverse disciplines to assess the state of the art in bone measurement.

Over 60 papers were presented at this meeting which covered absorptiometric methods and their applications, including recent developments in radiokinetic and radiographic techniques, Compton-scattering methods, neutron-activation analysis, and computed tomography. Particular emphasis was placed on the combined use of several methods.

An additional feature of this proceedings volume is an annotated bibliography of 303 non-clinical titles on bone and tissue measurement grouped under 11 subject headings to provide a convenient reference.

Publication of the PROCEEDINGS OF THE FOURTH INTERNATIONAL CONFERENCE ON BONE MEASUREMENT by the Institute through the Government Printing Office will make these research findings available to a wider audience to further interdisciplinary exchange and cooperation. Copies will be distributed to a worldwide readership of radiologists, radiation physicists, physicians, space scientists, instrument makers and equipment manufacturers and other investigators interested in bone measurement techniques.

Project Title: PROCEEDINGS OF THE WORKSHOP ON METHODOLOGIES FOR HUMAN POPULATION STUDIES IN NUTRITION RELATED TO HEALTH

Professional Personnel: Dr. Gerald F. Combs
Mrs. Marjorie C. Zukel
Mrs. Billie B. Mackey

Project Description:

This workshop on population studies in nutrition and the establishment of sound methodologies that are comparable among researchers in different countries was held July 24-25, 1979 at NIH in Bethesda, Maryland. Sponsored by the U.S.-Japan Malnutrition Panels of the U.S.-Japan Cooperative Medical Science Program including several NIH Institutes, it was attended by scientists from many countries.

The proceedings of the conference included presentations of both the American and Japanese speakers. Critical factors in epidemiologic studies and in obtaining reliable data on food intake and on health parameters were presented. The need was recognized for careful planning of studies in advance, for reliability tests for quality control, for collecting baseline data before carrying out an intervention study, and also that studies should fit into the population and cultural context of the subjects.

Anthropometric and laboratory measures in human nutrition studies were outlined, and criteria set forth for their selection. The value of measuring body length as well as weight and body composition in anthropometry was indicated.

Methodologies employed in the large national health surveys in Japan and the United States emphasizing the changing food habits in Japan and the probable result of these changes in the incidence of obesity in the population were described. Results of nutrition studies in Guatemala and Bangladesh were presented.

The proceedings also included an overview of osteoporosis, its prevalence in the population, the relationship to calcium intake, and methods of measuring bone mass.

Project Title: ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM TWELFTH ANNUAL CONTRACTORS' CONFERENCE PROCEEDINGS

Professional Personnel: Mrs. Billie B. Mackey

Man Years:

Total	.40
Professional	.20
Other	.20

Project Description:

The proceedings of the Twelfth Annual Contractors' Conference of the Artificial Kidney-Chronic Uremia Program held in Bethesda, Maryland on January 15-17, 1979 was published. Approximately 300 scientists from the United States and six foreign countries attended the conference including contractors and their staff, consultants to the Artificial Kidney-Chronic Uremia Program, interested scientists and Institute staff.

Presentations of existing contract-funded research and development projects were given by the investigators. Two workshops held on the first day were: "The Potential of Exercise in Maintenance Dialysis" and "Can Diet Be Dialysis Sparing?" Contracts on exercise training and diet therapy were presented during the workshops. Exercise training appears to alleviate complications such as hypertension and anemia, abnormalities in lipid, carbohydrate, and mineral metabolism in some dialysis patients. Diet therapy was considered both from cell metabolism and clinical aspects.

Studies comparing conventional hemodialysis with the newer approach to therapy known as hemofiltration were reported with the efficacy and costs of the two techniques compared and blood pressure response evaluated. Researchers reported on work to determine the long-term effects of continuous ambulatory peritoneal dialysis (CAPD). This treatment promises to be a simple and potentially less expensive form of therapy liberating the patient from the need to come to a dialysis center three times weekly for treatment. Studies are directed toward decreasing the rate of peritonitis, defining the ideal composition of the dialysate for this therapy, and improving the access devices through which dialysate is introduced into the abdominal cavity.

A plenary session included summaries of major points of the most significant findings given during the presentations.

The proceedings were given worldwide distribution to those with interests in the field of nephrology and artificial kidneys. It is available through the National Technical Information Service for a nominal fee.

Project Title: NATIONAL DIGESTIVE DISEASES EDUCATION AND INFORMATION CLEARINGHOUSE

Professional Personnel: Dr. Harold P. Roth
Mrs. Billie B. Mackey
Dr. Kirt J. Vener

Man Years:

Total	.3
Professional	.2
Other	.1

Project Description:

The goal of this project is to establish a National Digestive Diseases Education and Information Clearinghouse as recommended by the National Commission on Digestive Diseases in their report to the Congress dated January 31, 1979.

In their report to the Congress, the National Commission on Digestive Diseases recommended that a National Digestive Diseases Education and Information Clearinghouse be "established under NIAMDD to serve as a national educational resource for patients with digestive diseases and their families, physicians and other health professionals, and the general public, especially its neglected segments such as rural Americans, the elderly, minority groups, and children."

The Commission report recommended seven specific functions of the Clearinghouse that included: (1) the identification, collection, analysis, and dissemination of existing education and information materials relating to digestive health and diseases; (2) the evaluation of educational and informational materials by a panel of experts with regard to their accuracy, objectivity, and potential effectiveness, prior to their dissemination; (3) collaboration with representatives from the Office of Health Information and Health Promotion, and other Federal Organizations, with professional associations, with voluntary agencies, with the fields of education, business and industry, organized labor, and others to implement and coordinate a comprehensive, long-range public education and information program about digestive health and diseases; (4) the determination of needs for new educational and informational materials and programs, and subsequently the development of appropriate materials to meet the identified needs; (5) the production and dissemination of educational and instructional materials about digestive diseases to appropriate academic departments in medical schools in collaboration with the National Library of Medicine; (6) the development of cost effective means of disseminating updated information on a continuing basis to health professionals, educators, patients, and the general public about the existing materials and their sources; and (7) the development and distribution of a curriculum guide to digestive health and diseases for use in elementary and secondary schools.

In response to the Commission's recommendations, the Clearinghouse will consist primarily of a planned outreach educational and informational program with a clear, realistic focus and appropriate methods of evaluation. The Clearinghouse will be a central point for continuously collecting, evaluating, and classifying informational and educational materials, programs, and resources relevant to digestive health and diseases. Of primary importance, it will promote the exchange of information among the numerous foundations, voluntary health organizations, retirement groups, labor unions, HMO's and others with interests in digestive health and diseases. It will be the responsibility of the Clearinghouse to identify needed informational and educational materials and to encourage production of these needed materials. One of the prime functions of the Clearinghouse will be to act as a central point for providing information on available educational and informational materials to health professionals, doctors, nurses, medical assistants, patient care groups such as G.I. assistants, dietitians, endoscopic therapists, HMO's and others with interest in digestive diseases and health and as appropriate will provide the material.

ANNUAL REPORT SUMMARY
EPIDEMIOLOGY AND FIELD STUDIES BRANCH

The investigational activities of the Branch include epidemiological studies of diabetes, arthritis, digestive diseases and other metabolic diseases. In addition, the Branch gives epidemiological and biostatistical support to other components of the Intramural and Extramural programs of the Institute.

During the present year Dr. William Knowler was appointed Chief of the Southwestern Field Studies Section and Dr. Stephen Aronoff Chief of the Phoenix Clinical Research Section. Also, Dr. Timothy Kuberski has been appointed to the newly formed Epidemiology and Health Statistics Section of the Branch.

The major work of the Branch relating to the investigation of the distribution and pathogenesis of diabetes and obesity has been continued. Activities relating to arthritis epidemiology have been increased concomitant with the establishment of the Epidemiology and Health Statistics Section

Southwestern Field Studies Section

The Southwestern Field Studies Section has continued to investigate the determinants of diabetes and its complications primarily from the ongoing prospective study of the natural history of diabetes in the Pima Indian population. Investigation of genetic determinants of diabetes in the Pima Indian population has recently concentrated on the relationship of diabetes to HLA antigens. An association between the HLA-A2 antigen and diabetes has been described and evidence that the role of the HLA antigen is associated with determinants of the age of onset of diabetes has been found as allele frequencies were higher in younger diabetic subjects than in the older Pima Indian diabetics.

There have been further explorations of the role of obesity in diabetes and its relationship with parental diabetic status. While the phenotype of the parents and obesity are independent predictors of the risk of developing diabetes, the presence of parental diabetes appears to confer approximately a three-fold risk over that associated with the degree of obesity. The role of obesity on mortality rates in the Pima Indian population has also been investigated and, while excessive degrees of obesity are associated with increased mortality, more moderate degrees of obesity appear to be associated with lower mortality than even the absence of obesity.

Based on these data we have speculated that this provides further evidence for the "thrifty gene" hypothesis relating to the joint occurrence of diabetes and obesity in the population. Further evidence of the general validity of the "thrifty gene" hypothesis has also been obtained by documentation of an increasing prevalence of diabetes in the Pima population over a ten year period. The increased prevalence over time is also associated with an increased degree of obesity over the same time period. Furthermore review of the historical data on the Pima Indians and their frequency of diabetes and obesity in relationship to time strongly suggests that the frequency of diabetes has increased many-fold over the last 40 years.

The rate of development of micro- and macrovascular complications of diabetes has been examined in relation to baseline glucose levels and other possible determinants, such as hypertension. The relationship of these complications to fasting and two hour post load glucose levels has shown that there appears to be a threshold level of carbohydrate tolerance below which microvascular complications do not develop. The determination of this threshold has provided evidence of the validity of the new criteria for the diagnosis of diabetes. In contrast the development of electrocardiographic abnormalities and cardiovascular disease at autopsy shows a much more modest relationship with no evidence of a threshold value. These observations contribute to the definition of the nature of diabetes itself as well as pointing to a different pathogenetic mechanism for microvascular disease and atherosclerotic complications of the disease. The development of microvascular disease has shown also to relate to other factors such as the presence of hypertension, the control of which may ameliorate the rate of development of microvascular disease. Alternative evidence of the relationship between hyperglycemia and the development of retinopathy has been developed by use of glycosylated hemoglobin determinations and the fact that they, like fasting and two hour glucose concentrations, are highly correlated with the prevalence of retinopathy.

Data from the prospective study of glucose tolerance and its relationship to pregnancy outcome in the Gila River Indian population has resulted in quantification of the risks associated with various degrees of glucose intolerance to pregnancy outcome. Perinatal mortality rates, macrosomia, toxemia, and the rates of caesarean section have each been shown to vary directly with the glucose concentration measured in the third trimester of pregnancy, whereas congenital malformations have been shown to be restricted to the offspring of women known to have glucose intolerance at the onset of the pregnancy. The presence of glucose intolerance during the third trimester of pregnancy has been shown to be highly predictive of the development of diabetes mellitus in women in subsequent years.

Epidemiology and Health Statistics Section

Studies were undertaken to examine the frequency of certain seronegative arthropathies being observed clinically in Navajo Indians of northeast Arizona. The frequency of HLA-B27 and radiographic sacroiliitis was determined for the Navajo and compared to a group of Hopi Indians living in the same geographic area. HLA-B27 and radiographic sacroiliitis were found to be about three times more common in the Navajo than in the Hopi. HLA-B27 related forms of arthritis appear to be relatively common among the Navajo Indians. Additional studies are planned to examine the relationship of certain enteric bacterial pathogens and the development of arthritis in this population.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 69000-15 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Diabetes Mellitus in the Gila River Indian Community

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	P.H. Bennett	Chief	EFSB, NIAMDD
Other:	W.C. Knowler	Chief	SFSS, NIAMDD
	D.J. Pettitt	Staff Physician	SFSS, NIAMDD
	J.R. Lisse	Staff Physician	SFSS, NIAMDD
	E.J. Ballintine	Clinical Director	IRP, NEI
	D.L. Mann	Senior Investigator	IB, NCI

COOPERATING UNITS (if any) N.B. Rushforth, Case Western Reserve University; R. Kawate, Hiroshima University, Hiroshima, Japan; D. Doniach, Middlesex Hospital Medical School, London, U.K.; R.C. Williams, Arizona State University, B.S. Blumberg, Institute for Cancer Research, Philadelphia

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.5	4.0	4.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The natural history of the various manifestations and vascular complications of diabetes mellitus are being studied and evaluated in an Amerindian community. Various associated conditions and factors, including heredity, body weight, and blood pressure, that may have a bearing on the frequency and severity of diabetes and/or its complications are under investigation.

PHS-6040

Project Description:

Objectives:

1. Determine the prevalence and incidence of diabetes mellitus among those five years of age and over on the Gila River Indian Reservation.

2. Evaluate factors associated with the progression from prediabetic state to subclinical diabetes mellitus and to clinical diabetes.

3. Study risk factors associated with the rate of appearance and progression of vascular complications.

4. Investigate influence of tribe, age, obesity, parity and other factors on changes in glucose tolerance and their relationship to diabetes mellitus.

5. Determine the mode of inheritance of diabetes in the Pima Indians.

Methods Employed:

1. The following tests and examinations are repeated at two yearly intervals on all Indians five years of age or more living on the Sacaton Service Unit portion of the Gila River Reservation.

   a. Clinical interview
   b. Physical examination
   c. Modified glucose tolerance test and other biochemical tests

   In addition, on those aged 15 years and over:

   d. Fundus photographs
   e. Electrocardiogram
   f. Tests for vibratory sensation threshold
   g. Soft tissue radiographs for vascular calcification

2. Some of the above tests and examinations are also being done on Indians of other tribes or living under different environmental conditions and on non-Indian subjects for comparison.

3. Blood typing is performed on all subjects for red cell antigens, serum proteins, and Gm antigens. These typings provide an extensive set of approximately 40 gene markers for use in genetic analysis. In addition, selected individuals are HLA typed.

Major Findings:

1. Increased Incidence of Retinopathy in Diabetes with Elevated Blood Pressure. The incidence of retinopathy was assessed in 188 diabetic and 284 non-diabetic Pima Indian adults six years after an initial examination had shown each to be free of retinopathy. Examinations included direct ophthalmoscopy through dilated pupils. The incidence of retinal lesions was strongly related to the presence of diabetes and, among the diabetic subjects, to insulin treatment, disease duration, plasma glucose concentration, and presence of other complications such as proteinuria, loss of deep tendon reflexes, and increased vibration-sensation threshold. In diabetic subjects not taking insulin, the incidence of exudates in those with systolic blood pressures of at least 145 mm Hg was more than twice that of those with pressures of less than 125 mm Hg. This association persisted when assessed within categories of subjects stratified according to 13 potentially confounding variables, suggesting that control of blood pressure may reduce the incidence of retinal exudates in diabetics not treated with insulin.

2. Genetics of Diabetes Mellitus in Pima Indians. To study the association of diabetes with gene markers at the HLA locus, we HLA typed 191 unrelated diabetic Pima Indians ranging in age from 18 to 87 years and 69 unrelated non-diabetic Pima Indians at least 55 years old. The HLA-A2 antigen was found in 65% of the non-diabetic subjects and in 81% of the diabetic subjects (relative risk = 2.22, 95% confidence interval 1.21 - 4.06). Estimates of the HLA-A2 allele frequencies were 0.44 in the non-diabetic and 0.56 in the diabetic subjects. The HLA-A2 allele frequencies were inversely related to age at examination in the diabetic subjects (0.72 in those less than 35 years old, 0.59 in those 35-54 years old, and 0.52 in those at least 55 years old, test for linear trend in proportions, p=0.025). The decrease in the frequency of HLA-A2 with increasing age suggests that the HLA-A locus plays a role in the expression of diabetes, perhaps by contributing to an earlier age of onset.

3. Effects of Obesity and Parental Diabetes on Diabetes Incidence. The incidence of diabetes mellitus was determined in 3137 Pima Indians during periodic examinations that included measurement of weight, height, and glucose tolerance. Incidence was strongly related to preceding obesity, increasing steadily from 0.8±0.8 cases/1000 person-years in subjects with body mass index <20 kg/m^2 to 72.2±14.5 cases/1000 person-years (rate ± standard error) in those with body mass index ≥40 kg/m^2, when age-sex adjusted to the 1970 U.S. white population. There was little relationship between diabetes prevalence and concurrent obesity, illustrating the importance of longitudinal studies in estimating the effect of obesity

on the occurrence of a disease for which weight loss is a manifestation. The association of diabetes incidence with obesity remained within each group when subjects were classified by the diabetic status of their parents, another important risk factor for diabetes. Adjusted for age and obesity, incidence was 2.3 times as high (p=0.039) in subjects with one diabetic parent and 3.9 times as high (p=0.0003) in those with two diabetic parents as in those with two non-diabetic parents.

In the Pimas, both obesity and diabetes have become more common during this century, perhaps as a result of rapid cultural and dietary changes in a population genetically susceptible to diabetes. Similar increases in obesity and diabetes appear to be occurring in many other parts of the world.

4. Effects of Obesity and Diabetes on Mortality Rates. Mortality according to body mass index (weight/height^2, BMI) was studied in the Pima Indians, who are obese and have the highest known incidence of Type II diabetes mellitus. When age-sex adjusted to the Pima population aged 15-74 years, mortality was 14±1 deaths/1000 person-years (rate ± SE) in those with BMI <30 kg/m^2; slightly lower at 11±1 for BMI = 30-40 kg/m^2; but much higher at 26±8 for BMI ≥40 kg/m^2 (p=0.003 compared with BMI = 30-40 kg/m^2). The point at which increasing BMI was associated with greater mortality, equivalent to approximately 183% ideal weight from insurance statistics, is higher than in other populations. In non-diabetic Pimas the association of BMI and mortality was similar to that in the total population. However, mortality was more strongly related to BMI in diabetics, whose death rates were 24±7, 9±1, and 32±16 in those with BMI <30 kg/m^2, 30-40 kg/m^2, and ≥40 kg/m^2, respectively. The higher mortality among the lightest diabetics probably reflects weight loss with severe diabetes, rather than a direct effect of BMI on mortality. We have suggested that obesity had survival advantage for the Pimas because of their 2000 years of living in the desert with intermittent food supplies. Apart from conferring susceptibility to diabetes, obesity may be tolerated by Pimas except at the extreme of BMI ≥40 kg/m^2.

5. Relationships Between Fasting and Two-Hour Plasma Glucose and the Subsequent Incidence of Retinopathy and Heavy Proteinuria Confirm the New Diagnostic Criteria for Diabetes. The risk of developing microvascular complications over periods of three and six years, according to baseline plasma glucose concentrations, were estimated in Pima Indians who were at least 25 years of age. Retinopathy (microaneurysms, hemorrhages, or neovascularization) developed within three years in two per cent of 192 subjects with fasting plasma glucose concentrations less than 140 mg/dl and in 17 per cent of 99 subjects with higher initial fasting plasma glucose levels. Retinopathy developed within six years in two per cent of

457 subjects whose two-hour post-load glucose was less than 200 mg/dl and in 28 per cent of 187 subjects with higher levels. Heavy proteinuria (urine protein/creatinine ratio at least 1.0) was less frequent than retinopathy, but was similarly related to the initial fasting and two-hour plasma glucose concentrations. The finding that these microvascular complications rarely developed in individuals with fasting plasma glucose concentrations less than 140 mg/dl or two-hour plasma glucose concentrations less than 200 mg/dl supports the validity of the new diagnostic criteria for diabetes mellitus.

6. The Development of Retinopathy (Microvascular Disease) is More Strongly Related to Fasting or Post-Load Plasma Glucose Concentration than is Cardiovascular Disease (Macrovascular Disease). Pima Indians, at least 25 years old, with normal electrocardiograms (EKGs) and retinae, had plasma glucose concentrations determined two hours after 75 gms of oral carbohydrate at an initial exam. Six year incidence rates of retinopathy, defined as microaneurysms, hemorrhages, or neovascularization; and EKG changes, defined by U.S. Pooling Project Study criteria, were stratified by the plasma glucose.

   In 131 autopsies the presence of atherosclerotic cardiovascular disease (ASCVD), including old myocardial infarction, sudden death, and death due to stroke or myocardial infarction, was determined. Prevalence rates of ASCVD at death were stratified by the subjects' most recent two-hour plasma glucose.

   Retinopathy incidence increased steadily with two-hour plasma glucose, from 1.5% in people with <130 mg/dl to 48% with $\geq$ 500 mg/dl ($p<.0001$). In contrast, EKG changes and ASCVD at death showed little relationship with plasma glucose.

   Thus plasma glucose was a strong risk factor for the development of retinopathy (microvascular disease), but not for ASCVD (macrovascular disease).

7. Increasing Prevalence of Diabetes in the Pima Indians Over a Ten Year Period. A review of the literature suggests that both obesity and diabetes were much less frequent in the Pima Indians early in this century. This impression is difficult to confirm because systematic population studies of either condition were not made until the present longitudinal survey began in 1965. We estimated diabetes prevalence to be 24% (age-sex adjusted using the 1970 U.S. population as a standard) in the Pima Indians in 1967 and 34% in 1977, a 1.42-fold increase in ten years. During the same time period obesity increased in Pimas of almost all age and sex categories, which was probably an important contributory factor to the increase in diabetes prevalence.

8. The Glucose Tolerance Test, Even When Non-Diagnostic for Diabetes, Is Predictive of Subsequent-Overt Diabetes. We determined the six-year incidence of decompensation to a two-hour glucose concentration ≥300 mg/dl in 501 Pima Indians according to initial two-hour glucose concentration. The risk of decompensating increased with increasing initial two hour plasma glucose from 5.2% in those with glucose concentrations of less than 100 mg/dl to 81% of those with concentrations of 250 to 299 mg/dl. Thus the likelihood of decompensation of glucose tolerance over the six year period of followup was a direct function of the initial level of glucose tolerance. No particular glucose level was identified at which the risk of subsequent decompensation increased suddenly, but rather the risk increased steadily as a linear function over the whole range of initial glucose levels.

9. Glucosylated Hemoglobin Concentrations May be Useful in Identifying Diabetic Subjects, But Appear to Have No Advantage Over Fasting or Post Challenge Plasma Glucose Concentrations. Glycosylated hemoglobin (HBA_1) concentrations were determined in 300 Pima Indians aged 15 years and older. Frequency distributions of HbA_1 were unimodal in the 15-24 year-old age group, but were bimodal in those aged 25 years and over. The bimodality indicated that the subpopulation with diabetes could be identified by the presence of elevated HbA_1 levels. This group was comprised primarily of subjects who also had fasting plasma glucose levels of ≤140 mg/dl, but subjects with impaired glucose tolerance without fasting hyperglycemia had HbA_1 levels that were not significantly higher than those with normal glucose tolerance. The prevalence of diabetic retinopathy was much higher in the subgroup with elevated HbA_1 levels and increased with increasing HbA_1 level, although HbA_1 levels were no more strongly associated with the presence of retinopathy than were glucose concentrations. HbA_1 levels and triglyceride concentrations showed only a modest association. HbA_1 determinations provided no advantage over fasting or post challenge glucose levels in the diagnosis of diabetes.

Significance to Biomedical Research and the Program of the Institute: Elevated blood pressure was identified as a risk factor for retinal exudates, a form of diabetic retinopathy, in diabetics who were not treated with insulin. This may help in understanding the etiology of diabetic retinopathy, for which few risk factors other than diabetes itself are known. Other important risk factors for diabetic retinopathy were identified, including elevated plasma glucose concentration and therapy with insulin. The relationships of these variables need to be studied further. The finding that blood pressure is a risk factor for retinopathy in some diabetic patients has important therapeutic implications, because high blood pressure is potentially reversible by treatment, and thus the suggestion is made that some diabetic retinopathy might be preventable by blood pressure control. This should be tested by a clinical trial.

The HLA system has provided an important clue to studies of diabetes genetics, with the suggestion that either the HLA-A2 antigen, or the product of a gene in linkage disequilibrium with the HLA locus, plays a role in the genesis of the disease.

The strong effect of obesity on the development of diabetes has been further characterized and shown not to result simply from parental diabetes status. Because of weight loss occurring after development of the disease, there was little relationship between diabetes prevalence and concurrent obesity, illustrating the importance of longitudinal studies in estimating the effect of obesity on the development of diabetes.

The long-term experience with periodic glucose tolerance testing and assessment of microvascular diseases have demonstrated the usefulness of the glucose tolerance test in predicting the development of overt diabetes and its vascular complications. These findings have had an important impact on the adoption of standardized criteria for the diagnosis of diabetes. The finding that the effect of the glucose concentration on the development of macrovascular disease is much less than the effect on microvascular disease emphasizes the difference in causes of these two serious types of problems confronting diabetic patients.

Proposed Course: The long-term followup studies of diabetes and its vascular complications will continue. Surveillance of non-diabetic subjects becoming diabetic and of diabetics progressing with their disease gives an opportunity for further study of risk factors for diabetes and its complications. Further study of risk factors for diabetes will include studies of obesity, HLA type, and other gene markers. We will attempt further characterization of the mode of inheritance of diabetes in Pima Indians by studying segregation of the disease in pedigrees and linkage with HLA and other gene markers. Further studies of the role of blood pressure, plasma glucose concentration, and drug treatment on the incidence of diabetic retinopathy and nephropathy will be undertaken.

Publications:

1. Rushforth, N.B., Miller, M., and Bennett, P.H.: Fasting and two-hour post-load glucose levels for the diagnosis of diabetes. Diabetologia 16:373-379, 1979.

2. Knowler, W.C., Bennett, P.H., Bottazzo, G.F., and Doniach, D.: Islet cell antibodies and diabetes mellitus in Pima Indians. Diabetologia 17:161-164, 1979.

3. Savage, P.J., Hamman, R.F., and Bennett, P.H.: Prediabetes in Pima Indians. In Camerini-Davalos, R.A. (Ed.): Treatment of Early Diabetes. New York, Plenum Press, 1979, pp. 13-19.

4. Savage, P.J., Bennett, P.H., Senter, R.G., and Miller, M.: High prevalence of diabetes in young Pima Indians: evidence of phenotypic variation in a genetically isolated population. Diabetes 28:937-942, 1979.

5. Bennett, P.H., Knowler, W.C., Rushforth, N.B., Hamman, R.F., and Savage, P.J.: The Role of Obesity in the Development of Diabetes in the Pima Indians. In Vague, J. and Vague, Ph. (Eds.): Diabetes and Obesity. Amsterdam, Excerpta Medica, 1979, pp. 117-126.

6. Bennett, P.H., and Savage, P.J.: Prognostic Factors for the Development of Diabetes Mellitus in Pima Indian Children and Young Adults. In Grave, G. (Ed.): Early Detection of Potential Diabetics: The Problems and the Promise. New York, Raven Press, 1979, pp. 53-67.

7. Flock, E.V., Bennett, P.H., Savage, P.J., Webner, C.J., Howard, B.V., Rushforth, N.B., and Miller, M.: Bimodality of glycosylated hemoglobin distribution in Pima Indians: relationship to fasting hyperglycemia. Diabetes 28:984-989, 1979.

8. National Diabetes Data Group: Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. Diabetes 28:1039-1057, 1979.

9. Knowler, W.C., Bennett, P.H., and Ballintine, E.J.: Increased incidence of retinopathy in diabetics with elevated blood pressure. A six-year followup study in Pima Indians. New Engl. J. Med. 302:645-650, 1980.

10. Bennett, P.H., and Knowler, W.C.: Increasing Prevalence of Diabetes in the Pima (American) Indians Over a Ten Year Period. In Waldhausl, W.K. (Ed.): Diabetes 1979. Amsterdam, Excerpta Medica, 1979, pp. 507-511.

11. Craig, F., Lustbadar, E.D., Merten, C., and Blumberg, B.S.: Serum iron levels and response to hepatitis B virus. Proc. Natl. Acad. Sci. U.S.A. 76:2438-2441, 1979.

12. Kuberski, T.T., and Bennett, P.H.: Diabetes mellitus as an emerging public health problem on Guam. Diabetes Care 3:235-241, 1980.

13. Kuberski, T.T., and Bennett, P.H.: The status of diabetes mellitus in the territory of Guam. South Pacific Commission Information Document No. 47. Noumea, New Caledonia, 1979., 48 pp.

14. Bennett, P.H., Knowler, W.C., Pettitt, D.J., and Lisse, J.R.: The prognostic significance of the glucose tolerance test. Proc. of the VIth International Congress of Endocrinology, Melbourne (in press), 1980.

15. Knowler, W.C., Pettitt, D.J., Savage, P.J., and Bennett, P.H.: Diabetes incidence in Pima Indians: contributions of obesity and parental diabetes. Am. J. Epidemiol. (in press), 1980.

16. Bennett, P.H.: Diabetes and Heart Disease - the Magnitude of the Problem. In Scott, R.C. (Ed.): Diabetes and the Heart (in press).

NIH Contract No. NO1-AM-6-2221 Starting Date: September 30, 1979
Expiration Date: September 29, 1980

Contractor: Case Western Reserve University

Money Allocated: $98,012 Man Years Purchased: 1.9

Pathogenesis of Diabetes Mellitus in the Gila River Indian Community

Some of the development of the mathematical theory and computation and analysis involved in the above project was carried out under this contract with Case Western Reserve University, Cleveland, Ohio. Methodology was further developed for grouping subjects by characteristics which are bimodally distributed, and for describing relationships between glucose tolerance and various measures of obesity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69001-11 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Complications and Outcome of Diabetic and Prediabetic Pregnancies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter H. Bennett	Chief	EFSB	NIAMDD
	William C. Knowler	Chief	SFSS	NIAMDD
	David J. Pettitt	Staff Physician	SFSS	NIAMDD
	Jeffrey R. Lisse	Staff Physician	SFSS	NIAMDD

COOPERATING UNITS (if any)
Indian Health Service
Dr. Kirk A. Aleck, Dept. of Pediatrics, University of New Mexico

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To determine the effects of diabetes mellitus and prediabetes on the complications and outcome of pregnancy in women in the Gila River Indian Community.

The characteristics of all pregnancies among residents of the Gila River Indian Community will be determined. Each pregnant woman will have a modified glucose tolerance test during the third trimester and following delivery and will be retested at approximately two yearly intervals in conjunction with the prospective study of diabetes mellitus in the same community. The characteristics of the pregnancies in women who subsequently become diabetic will be compared to those among women who were diabetic at the time of pregnancy and those who remain nondiabetic.

PHS-6040

Project Description:

Objectives: To document the natural history and to determine the effect of diabetic and prediabetic pregnancies in a Pima Indian group with the highest recorded prevalence of diabetes mellitus. Special emphasis will be placed on complications during pregnancy and associations with mortality and morbidity in the offspring.

Methods Employed: The course and outcome of pregnancies in all female residents of the Gila River Indian Community (Sacaton Service Unit area) are followed and recorded in a standardized manner.

All patients attending prenatal clinic at Sacaton Hospital receive a modified glucose tolerance test during the third trimester of their pregnancy. These patients receive another modified glucose tolerance test 24-48 hours post partum and a chart review and patient interview are done for additional information. They are retested at approximately 2 yearly intervals to document the development or not of diabetes. They will also be retested for each subsequent pregnancy.

Infants will be examined to determine the presence of congenital anomalies and data related to mortality and morbidity will be collected and documented. At age five years, the children will be re-examined to detect any further abnormalities which may have been inapparent at birth.

Major Findings: A modified oral glucose tolerance test was done during the third trimester in 811 pregnancies in Pima Indian women over a 13-yr period, and maternal and fetal complications were documented. Diabetes was known to be present in 51 pregnancies. Among those who were not previously known to have diabetes, rates of perinatal mortality, macrosomia, toxemia, and cesarean section varied directly with glucose concentration, but congenital malformation and prematurity rates did not. Rates of all of these complications were higher in known diabetic women than in the remainder of the population. In addition to glucose concentrations, maternal weight and age were predictive of macrosomia and toxemia. Third-trimester glucosuria was found to be of very limited value as a screening procedure for gestational diabetes. In 233 women followed for 4-8 yr, the third-trimester glucose concentration was highly predictive of the subsequent incidence of diabetes.

Significance to Biomedical Research and the Program of the Institute: In view of the high prevalence of diabetes in the Pima Indians, studies in the Gila River Community provide a unique opportunity to evaluate the outcome of both prediabetic pregnancies and pregnancies with mild carbohydrate intolerance. Retrospective studies of the course of prediabetic pregnancies have to date produced conflicting results. Although it is well established that patients with severe glucose intolerance, especially those with diabetes of long duration and significant microvascular complications, have an increased prevalence of fetal mortality and maternal morbidity, it is not certain whether treatment will improve the outcome of those pregnancies in

which there is mild glucose intolerance. Limited prospective studies of the benefit of treating pregnant women with mild glucose intolerance have so far yielded conflicting results. With the adoption of more strigent criteria for the treatment of mild carbohydrate intolerance in pregnancy it is hoped that this study will be able to add to the evaluation of this problem by monitoring the impact of these therapeutic changes on the outcome of pregnancies on the reservation.

Proposed Course: The pregnancy study will continue in conjunction with the study of the natural history of diabetes mellitus in the Gila River Indian Community. The number of pregnancies recognized to be prediabetic will increase as the mothers studied develop diabetes mellitus.

Publications:

Pettitt, D.J., Knowler, W.C., Baird, H.R., and Bennett, P.H.: Gestational diabetes: infant and maternal complications of pregnancy in relation to third-trimester glucose tolerance in Pima Indians. Diabetes Care 3:458-464, 1980.

Collaboration with Project No. 78-CH-102 entitled "Breast Feeding Among the Pima Indians: Time Trend, Factors Associated with Breast Feeding, and its Effect on Child Health." (Principle Investigator Dr. Michele Forman, NICHHD).

The staff of the SFSS, NIAMDD collaborated in the above project which resulted in the following major findings: Among women now at least 35 years old, there was a significant decline over time in the proportion breast feeding between 1950 and 1977, whereas the proportion of younger women breast feeding increased during this interval. All women who breast fed had attended prenatal clinic, and on average started attending prenatal clinic earlier in their pregnancies than women who did not breast feed. Social and demographic variables associated with breast feeding are still under study.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 69006-10 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Gila River Indian Community Autopsy Study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	P.H. Bennett	Chief	EFSB, NIAMDD
Other:	W.C. Knowler	Chief	SFSS, NIAMDD
	D.J. Pettitt	Staff Physician	SFSS, NIAMDD
	J.R. Lisse	Staff Physician	SFSS, NIAMDD

COOPERATING UNITS (if any) D. Kuban, Chief, Pathology Dept., Phoenix Indian Medical Center; P. LeCompte, Clinical Professor Emeritus, Harvard Medical School; L. Klein, Professor of Orthopedic Surgery and Biochemistry; Case Western Reserve Univ.; L. Orci, Chairman, Dept. of Morphology, University of Geneva

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.2	.4

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The postmortem characteristics of Pima Indians of the Gila River Indian Community are investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects.

Postmortem examinations are performed on members of the Gila River Indian Community whenever possible, regardless of whether the subjects have prior evidence of diabetes. Tissues are examined by light and electron microscopy and the histology related to the clinical findings in the same subjects. The histology of the pancreatic islets of Langerhans is being studied in detail to describe the changes that take place in diabetes. The distribution of cell types varies considerably between different regions of the pancreas suggesting regional variation in function.

Project Description:

Objectives:

1. To determine the causes of death, and associated disease processes in members of the Gila River Indian Community being followed in prospective studies on arthritis, diabetes mellitus, cholelithiasis, etc.

2. To ascertain and evaluate the histopathological changes in tissues and cells as they relate to diabetes mellitus. To assess their importance and relationship to clinical findings in members of the Gila River Indian Community upon whom autopsies are performed.

3. Evaluate factors and conditions associated with increased or decreased mortality rate in the Gila River Indians.

Major Findings: Studies of the histology of the Islets of Langerhans in human pancreases revealed a gross heterogeneity of the distribution of the diffused cell types. This implies that uniform sampling methods and sensitive quantitative analysis of cell distributions are necessary for comparative studies.

Preliminary lipoprotein analyses of 13 Pima Indians that died of cardiovascular causes and 13 age-sex, weight, and diabetic status matched controls that died of other causes suggested that a low ratio of high density lipoprotein cholesterol to total serum cholesterol was associated with increased mortality.

Over a hundred autopsy cases were screened for cardiovascular disease. Forty males 55 years of age or older were selected and the prevalence of cardiovascular disease at autopsy was stratified by the most recent 2 hour post load plasma glucose. An approximate doubling of the prevalence of cardiovascular disease at autopsy was seen when comparing those with glucose concentrations greater than or equal to 200 mg/dl with those less than 200 mg/dl.

Significance to Biomedical Research and the Program of the Institute: As more subjects who have been under long term ante-mortem surveillance are autopsied, more precise clinical pathologic correlations will be available for the study of diabetes mellitus than has previously been possible.

Proposed Course: The systematic collection and cataloging of tissues will continue. As sufficient specimens become available, detailed examinations of various tissues will be made. Further studies of the kidney, including electron microscopy and biochemical quantification of the insulin-glucagon, and pancreatic polypeptide producing cells of the Islets of Langerhans will be the major activity in the coming year.

Publications: None

Z01 AM 69006-10 EFS

NIH Contract No. N01-AM-7-2213 Starting Date: September 1, 1979
Expiration Date: August 31, 1980

Contractor: University of Geneva, Geneva, Switzerland

Money Allocated: #32,400 Person-years Purchased: 1

Conducting Morphological Studies of Diabetes

Under this contract, the detailed morphology of pancreases from Pima autopsy specimens is being studied. The different cell types of the Islets of Langerhans are being quantified using immunofluorescent techniques. Human fibroblasts isolated from diabetic and non-diabetic Pimas will be compared for morphologic differences.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 69009-15 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Natural History of Arthritis and Rheumatism on the Gila River Indian Community

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	P.H. Bennett	Chief	EFSB, NIAMDD
Other:	J.R. Lisse	Staff Physician	SFSS, NIAMDD
	D.J. Pettitt	Staff Physician	SFSS, NIAMDD
	W.C. Knowler	Chief	SFSS, NIAMDD
	T.T. Kuberski	Medical Officer	EHSS, NIAMDD

COOPERATING UNITS (if any)
Dean Mann, Immunology Branch, National Canter Institute

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.7	.2	.5

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The development and progression of various forms of arthritis is being investigated in an American Indian populations. The patterns of joint disease will be examined to determine if distinctive patterns of degenerative joint disease can be recognized, and if so, their evolution and outcome will be followed: In addition, the prognostic significance of various factors related to rheumatoid arthritis and ankylosing spondylitis will be examined.

The study which includes clinical radiographic and serologic studies of joint disease is a part of a prospective study of the Gila River Indian Community in which studies of diabetes mellitus and other diseases are being undertaken.

Project Description:

Objectives:

1. Determine the progression of clinical, radiological and serological features of joint diseases affecting the Gila River Indians with particular attention to rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and gout.

2. Determine environmental and genetic factors associated with development and progression of arthritis and rheumatism.

3. To compare and contrast the clinical course of rheumatoid arthritis in sero-positive individuals with that of sero-negative arthritics.

4. Determine the predictive and prognostic significance of rheumatoid factor in relation to the clinical course of rheumatoid arthritis.

Methods Employed:

1. The following tests and examinations will be repeated on all Indians five years of age and over, living on the Sacaton Service Unit portion of the Gila River Reservation.

   a. Clinical interview
   b. Physical examination
   c. Radiographs of hands and feet. Males over age 17 and females over age 44 will have a pelvis film taken
   d. Laboratory determinations: rheumatoid factor, uric acid

2. The data from the above examinations and tests as well as demographic pedigree, environmental information, and data on other conditions and diseases will also be collected.

3. HLA and DR typing are performed on subject with rheumatoid arthritis, ankylosing spondylitis and appropriate controls.

Major Findings: Studies of HLA-B27 positive and negative ankylosing spondylitis has shown as increased prevalence of sacroiliitis in older males with HLA-B27. Other literature has suggested that 20% of those with HLA-B27 will eventually develop sacroiliitis. Our features indicate 55% of our B27 subjects had radiographic sacroiliitis. We are in the process of freezing lymphocytes and looking for environmental triggers of the disease in these people.

Significance of Biomedical Research and the Program of the Institute: Recently the recognition of association between the DR locus and inflammatory arthritis has reopened the search for genetic determinants and determinants for susceptibility to these diseases. Examination of these associations in subjects of different racial backgrounds may lead to the identification of

specific determinants of these diseases. Environmental trigger factors have long been suspected in autoimmune disease, but recent recognition increased numbers of Klebsiella in the stools of spondylitics, and interactions with their lymphocytes have made this a renewed area of interest, and may lead to more clues about the pathogenesis of ankylosing spondylitis. Our population, with the high prevalence of sacroiliitis, makes them ideal for such studies.

Proposed Course: Collaborative efforts to define B lymphocyte associated antigens and ankylosing spondylitis and rheumatoid arthritis will be continued.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 69012-02 EFS

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Descriptive Epidemiology of Glomerulonephritis and Other Urinary Disorders

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Jeffrey A. Cutler Epidemiologist EFS, NIAMDD

COOPERATING UNITS (if any) Missouri Kidney Program, University of Missouri, Columbia,Mo.
Department of Medical Statistics and Epidemiology, Mayo Clinic
Office of Biometry and Field Studies, NINCDS, NIH
Various divisions, National Center of Health Statistics

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project continues the study of the epidemiology of renal and urinary diseases using previously collected data from death certification and health surveys. In addition, new data collection from medical records was planned and initiated. Specific questions raised by earlier work regarding variation in disease prevalence and mortality were addressed.

Project Description:

Objectives: To estimate the incidence, prevalence, and/or mortality of glomerulonephritis, proteinuria, and other urinary conditions with emphasis on causes of end-stage renal failure, and to draw inferences on the cause and course of these conditions. The work has shifted from a descriptive to an explanatory emphasis during this year.

Methods Employed: Studies of glomerulonephritis have gone beyond standard diagnostic coding for case selection to examine diagnostic terms and their criteria. Data on expenditures for treatment of end-stage renal failure have been utilized in the analysis of variation in mortality.

Major Findings: The national financing of treatment for end-stage renal disease under Medicare appears to be one explanation for declining mortality, but not the major one. Hospital data coded under ICDA-8 to the rubric for glomerulonephritis largely represents unrelated acute renal failure.

Significance: Glomerulonephritis is the cause of most end-stage renal failure, and its etiology is largely unknown. The report, Research Needs in Nephrology and Urology (Annual Report, 1977-78, p. 1044), emphasized research on this group of diseases, including its epidemiology and possible viral causes.

Proposed Course: It is likely that this project will be largely inactive during the next fiscal year.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM70500-02 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on Insulin's Regulation of Glucose Metabolism by Adipose Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lester B. Salans	Chief	CMOS, NIAMDD
	Samuel W. Cushman	Associate Chief	CMOS, NIAMDD
OTHER:	Elizabeth A.H. McGuire	Research Chemist	CMOS, NIAMDD
	Paul J. Hissin	Staff Fellow	CMOS, NIAMDD
	Eddy Karnieli	Visiting Fellow	CMOS, NIAMDD
	Ian A. Simpson	Visiting Associate	CMOS, NIAMDD

COOPERATING UNITS (if any) Department of Biochemistry, Dartmouth Medical School; Diabetes Branch, NIAMDD, NIH; Laboratory of Metabolism, NIAAA, ADAMHA; Institute of Medical Research, University of Geneva, Switzerland

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Cellular Metabolism and Obesity

INSTITUTE AND LOCATION
NIAMDD, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.50	5.50	3.00

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Elucidation of the mechanism of insulin's stimulatory action on glucose metabolism and its perturbation in obesity and with altered dietary carbohydrate and fat content has been undertaken using the isolated rat adipose cell as a model. Mathematical modeling of the complex kinetics of insulin binding is in progress. A specific D-glucose-inhibitable ^{3}H-cytochalasin B binding assay for quantitating the number of glucose transport systems in subcellular membrane fractions has been developed and a large intracellular pool of glucose transport systems identified in association with the Golgi fraction. Detailed kinetic, reversal, and insulin concentration experiments further establish that insulin appears to stimulate glucose transport by triggering a rapid and reversible translocation of glucose transport systems from this intracellular pool to the plasma membrane. Studies in enlarged adipose cells from obese rats further suggest that increased basal glucose transport and the markedly diminished transport response to insulin are accounted for by an insulin-like redistribution of transport systems even in the basal state. An examination of the quantitative relationship between insulin binding and the stimulation of glucose transport are also in progress.

Objectives: Insulin's ability to stimulate the uptake and metabolism of glucose by such peripheral tissues as muscle and adipose tissue and to inhibit lipolysis in the latter tissue plays a major role in systemic glucose and lipid homeostasis in vivo in man and experimental animal. Insulin resistant states, exemplified by obesity and noninsulin-dependent diabetes mellitus, are accompanied, in turn, by perturbed systemic glucose and lipid homeostasis and a reduction in the capacity of the peripheral tissues to respond to insulin. Increasing ratios of dietary carbohydrate to fat, on the other hand, tend to ameliorate these insulin resistant states. The alterations in peripheral tissue function observed in vivo in these altered physiological and pathophysiological metabolic states are closely paralleled by similar alterations in adipose and muscle cell function studied in vitro. The overall objective of the Cellular Metabolism and Obesity Section is an elucidation of the mechanism of insulin action and its perturbation in obesity and diabetes and with altered dietary carbohydrate and fat content. Over the past several years, this objective has been approached through a detailed examination of the isolated adipose cell as a model system. The Section's specific objectives comprise the three major projects now presently in progress: 1) mechanism of insulin action on the adipose cell, 2) characterization of the glucose transport system in the adipose cell, and 3) alterations in adipose cell function in the aging rat model of obesity. Two additional major projects are temporarily inactive: 1) alterations in adipose cell function in the genetically obese Zucker fatty rat, and 2) role of dietary carbohydrate and fat in regulating rat adipose cell function.

Progress: Elucidation of the mechanism of insulin's stimulatory action on glucose metabolism and its perturbation in obesity and with altered dietary carbohydrate and fat content has been undertaken using the isolated rat adipose cell as a model. Mathematical modeling of the complex kinetics of insulin binding is in progress. A specific D-glucose-inhibitable ^{3}H-cytochalasin B binding assay for quantitating the number of glucose transport systems in subcellular membrane fractions has been developed and a large intracellular pool of glucose transport systems identified in association with the Golgi fraction. Detailed kinetic, reversal, and insulin concentration experiments further establish that insulin appears to stimulate glucose transport by triggering a rapid and reversible translocation of glucose transport systems from this intracellular pool to the plasma membrane. Studies in enlarged adipose cells from obese rats further suggest that increased basal glucose transport and the markedly diminished transport response to insulin are accounted for by an insulin-like redistribution of transport systems even in the basal state. An examination of the quantitative relationship between insulin binding and the stimulation of glucose transport are also in progress.

Insulin's complex interaction with specific binding sites in the plasma membrane of the adipose cell is accompanied by a rapid, concentration-dependent stimulation of glucose transport into, and glucose metabolism by, this cell. The mechanism of these actions of insulin are under investigation through 1) the development of a mathematical model of ^{125}I-insulin binding, and 2) studies of the relationship between insulin binding and glucose transport activity under perturbed experimental conditions. To date, a one binding site model of insulin's interaction with the adipose cell has been rejected although some of the kinetics of insulin association can be successfully explained by the hormone degradation portion of this model. While a two binding site model can explain the kinetics of both association and dissociation at a given insulin concentration, the parameters of each site or the proportion of sites with constant parameters must vary with insulin concentration. A variety of experimental conditions have been found which appear to differentially affect insulin binding and insulin's ability to stimulate glucose transport, thereby providing several potential means of identifying that specific aspect of insulin-receptor interaction leading to enhanced glucose transport.

Glucose transport occurs in the adipose cell through a highly specific facilitated diffusion process. Insulin, in turn, increases the maximum transport velocity. A specific D-glucose inhibitable ^{3}H-cytochalasin B binding assay has recently been used to quantitate the number of glucose transport systems in the purified plasma membranes of isolated rat adipose cells and to demonstrate that insulin stimulates glucose transport primarily by increasing the number of functional glucose transport systems. This assay has now been further used to identify a high specific activity intracellular pool of transport systems which sediments with the Golgi fraction during differential ultracentrifugation. Insulin, in turn, stoichiometrically decreases the number of transport systems in this pool at the same time that it increases their number in the plasma membranes. These effects of insulin are rapid, correlating in time with insulin's stimulatory action on glucose transport itself in the intact cell, and completely reversible by treating the insulin-stimulated cell with excess insulin antibody, suggesting a translocation process. Preliminary results suggest that insulin may stimulate glucose transport in the rat diaphragm in vitro by the same mechanism.

Increasing adipose cell size in the aging rat model of obesity is accompanied by an increasing basal glucose transport activity and a decreasing ability of insulin to stimulate glucose transport activity in the intact isolated adipose cell. The ability of insulin to increase the number of functional glucose transport systems in this cell's plasma membranes, as assayed by D-glucose-inhibitable ^{3}H-cytochalasin B binding, is also decreased. Since insulin appears to enhance glucose transport by triggering the translocation of transport systems to the plasma membrane from an intracellular pool which is associated with a fraction enriched in membranes of the Golgi apparatus, the effects of cell size on the number of transport systems in this pool and the

total number of transport systems per cell have been examined. The markedly enlarged cells prepared from 800 g rats contain, at most, 50% more transport systems per cell than small cells from 180 g rats. Furthermore, even in the basal state, the number of transport systems in the enlarged cell's plasma membranes is already increased and this cell's intracellular pool is already depleted. Insulin has little further effect on the distribution of transport systems.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM70501-02 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanism of Insulin Action on the Adipose Cell

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elizabeth A.H. McGuire	Research Chemist	CMOS, NIAMDD
OTHER:	Paul J. Hissin	Staff Fellow	CMOS, NIAMDD
	Samuel W. Cushman	Associate Chief	CMOS, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Cellular Metabolism and Obesity

INSTITUTE AND LOCATION
NIAMDD, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.30	1.10	0.20

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin's complex interaction with specific binding sites in the plasma membrane of the adipose cell is accompanied by a rapid, concentration-dependent stimulation of glucose transport into, and glucose metabolism by, this cell. The mechanism of these actions of insulin are under investigation through 1) the development of a mathematical model of ^{125}I-insulin binding, and 2) studies of the relationship between insulin binding and glucose transport activity under perturbed experimental conditions. To date, a one binding site model of insulin's interaction with the adipose cell has been rejected although some of the kinetics of insulin association can be successfully explained by the hormone degradation portion of this model. While a two binding site model can explain the kinetics of both association and dissociation at a given insulin concentration, the parameters of each site or the proportion of sites with constant parameters must vary with insulin concentration. A variety of experimental conditions have been found which appear to differentially affect insulin binding and insulin's ability to stimulate glucose transport, thereby providing several potential means of identifying that specific aspect of insulin-receptor interaction leading to enhanced glucose transport.

Objectives: Insulin's hypoglycemia effect in man and experimental animal *in vivo* has long been recognized, as has been insulin's ability to stimulate the uptake and metabolism of glucose by such peripheral tissues as muscle and adipose tissue. Indeed, insulin's effects on peripheral tissue glucose metabolism *in vivo* are closely paralleled by similar effects on the metabolism of glucose by isolated muscle fibers and adipose cells *in vitro*. While adipose tissue and its constituent adipose cells probably do not represent a primary site of glucose utilization in overall glucose homeostasis in the intact organism, except perhaps in the young and rapidly growing animal, its availability and the homogeneity of the cells prepared therefrom render this tissue an ideal model system with which to examine the mechanism(s) of insulin action at the cellular and subcellular levels. The overall objective of the present investigation is to elucidate the mechanism through which insulin regulates glucose metabolism in the rat, and ultimately human, adipose cell. This mechanism comprises a complex interaction of insulin with specific binding sites in the adipose cell's plasma membrane, a rapid stimulation of glucose transport into the adipose cell, and a subsequent enhancement of the activity of a variety of specific pathways for glucose carbon metabolism in the adipose cell. Insulin also inhibits the stimulation of the adipose cell's lipolytic activity by epinephrine and interacts with this lipolytic hormone in regulating glucose carbon metabolism. The specific objectives for the current year have been to continue 1) the development of a mathematical model of the kinetics of ^{125}I-insulin association with, dissociation from, and degradation by, the isolated rat adipose cell, and 2) studies of the quantitative relationship between insulin binding to, and insulin's stimulatory action on glucose transport activity in, the isolated rat adipose cell under experimental conditions where these two functions might be partially dissociated.

Methods: In all experiments, isolated adipose cells were prepared by incubating the whole epididymal fat pads of male Sprague Dawley rats weighing 150-200 µg with collagenase, separating the liberated adipose cells from the stromal-vascular elements by thorough washing, and resuspending the washed cells as a stock cell suspension. Adipose cell size and concentration in the stock cell suspension were determined using an osmic acid fixation, Coulter Electronic Counter method.

In the first series of experiments, the kinetics of ^{125}I-insulin association was then examined by incubating samples of adipose cells from the stock cell suspension for varying periods of time up to 300 minutes at temperatures varying from 15-37°C in the presence of tracer ^{125}I-insulin and 0-17 µM (2.5 U/ml) native insulin. The dissociation of bound ^{125}I-insulin was examined by adding additional native insulin at varying concentrations up to 17 µM (2.5 U/ml) and continuing the incubation for varying additional periods of time up to 300 minutes. The incubated cells were assayed for bound ^{125}I-insulin

and the incubation medium, for degraded ^{125}I-insulin. The results of these experiments were analyzed using the SAAM27 compartmental modeling program implemented on an IBM 1108 computer at the National Bureau of Standards.

In the second series of experiments, the relationship between steady state insulin binding and insulin's concentration-dependent stimulation of glucose transport activity were then examined together in samples of adipose cells from the stock cell suspension under varying experimental conditions by well established techniques. An initial variable of interest was pH. Insulin binding at pH 6.0-9.0 was measured by incubating cells for 90 minutes at 24°C or for 30 minutes at 37°C in the presence of tracer ^{125}I-insulin and increasing concentrations of native insulin up to 17 μM (2.5 U/ml). Glucose transport activity over the same pH range was estimated by preincubating cells for 30 minutes at 37°C in the presence of 0.35 nM (0-5000 μU/ml) insulin, then measuring the time-dependent uptake of 0.1 mM ^{14}C-3-O-methylglucose, a nonmetabolizable glucose analogue transported exclusively by the glucose transport system. Analysis of the resulting binding and transport isotherms was undertaken by a curve fitting procedure.

Results and Discussion: Tracer ^{125}I-insulin rapidly associates with the isolated rat adipose cell in a time- and temperature-dependent manner. At lower incubation temperatures, tracer binding ultimately attains a steady state level. With increasing incubation temperature, however, insulin degradation increases such that at 37°C tracer binding reaches an early maximal level, then declines. When high concentrations of native insulin are added to the cells during the association period, further tracer binding is prevented and that tracer already bound to the cells rapidly dissociates. The rate of dissociation appears to depend upon the concentration of native insulin added, the duration of the association period, and the incubation temperature. A one binding site mathematical model of this complex process cannot satisfactorily account for the observed results although the degradation portion of this model does appear to explain the decrease in tracer association with longer incubation periods at higher temperatures. A two site mathematical model of insulin binding has, however, now been formulated which can explain both insulin association and dissociation at a given insulin concentration. This model permits estimation of the rate constants for each site with reasonable certainty, as well as the proportion of the total number of sites in each class. Nevertheless, a simple two site model cannot account for the relationship between insulin binding and insulin concentration. Two variations in the model are, therefore, under consideration: one in which the proportion of sites in each class varies with horone concentration, and one in which the rate constants vary. These variations are now being tested.

Steady state concentration-dependent insulin binding and insulin's concentration-dependent stimulation of glucose transport activity appear, in preliminary experiments, to be differentially affected by the pH at which these two activities are measured. Further detailed studies of these pH effects are in progress as are initial attempts to examine the observed binding and transport isotherms with a curve fitting technique.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM70502-02 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Characterization of the Glucose Transport System in the Adipose Cell

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Samuel W. Cushman	Associate Chief	CMOS, NIAMDD
OTHER:	Eddy Karnieli	Visiting Fellow	CMOS, NIAMDD
	Ian A. Simpson	Visiting Associate	CMOS, NIAMDD
	Paul J. Hissin	Staff Fellow	CMOS, NIAMDD
	Lester B. Salans	Chief	CMOS, NIAMDD

COOPERATING UNITS (if any) Department of Biochemistry, Dartmouth Medical School; Diabetes Branch, NIAMDD, NIH; Laboratory of Metabolism, NIAAA, ADAMHA; Institute of Medical Research, University Geneva, Switzerland

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Cellular Metabolism and Obesity

INSTITUTE AND LOCATION
NIAMDD, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.70	3.00	1.70

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Glucose transport occurs in the adipose cell through a highly specific facilitated diffusion process. Insulin, in turn, increases the maximum transport velocity. A specific D-glucose inhibitable ^{3}H-cytochalasin B binding assay has recently been used to quantitate the number of glucose transport systems in the purified plasma membranes of isolated rat adipose cells and to demonstrate that insulin stimulates glucose transport primarily by increasing the number of functional glucose transport systems. This assay has now been further used to identify a high specific activity intracellular pool of transport systems which sediments with the Golgi fraction during differential ultracentrifugation. Insulin, in turn, stoichiometrically decreases the number of transport systems in this pool at the same time that it increases their number in the plasma membranes. These effects of insulin are rapid, correlating in time with insulin's stimulatory action on glucose transport itself in the intact cell, and completely reversible by treating the insulin-stimulated cell with excess insulin antibody, suggesting a translocation process. Preliminary results suggest that insulin may stimulate glucose transport in the rat diaphragm in vitro by the same mechanism.

Objectives: Insulin's ability to stimulate the uptake and metabolism of glucose by such peripheral tissues as muscle and adipose tissue has long been recognized as one of the primary mechanims through which this hormone contributes to systemic glucose homeostasis in vivo in both man and experimental animal. More recently, glucose transport activity has been implicated as the rate limiting step for glucose metabolism in the adipose, and probably muscle, cell under most physiological conditions. The entry of glucose into the adipose cell is mediated by a highly specific facilitated diffusion process. Insulin's stimulatory action on glucose uptake and metabolism in this cell occurs, in turn, primarily through an increase in the maximum transport velocity, either by increasing the mobility of pre-existing transport systems which are functional in the absence of insulin, by increasing the total number of transport systems which continue to function at a constant mobility, or by some combination of the two. The overall objective of the present investigation is to characterize the adipose cell's glucose transporter proteins and elucidate the process through which their activities are modulated by insulin. Previously, a specific D-glucose-inhibitable equilibrium ^{3}H-cytochalasin B binding assay has been developed for quantitating the number of glucose transport systems in the purified plasma membranes of isolated rat adipose cells and used to demonstrate that insulin stimulates glucose transport in the adipose cell primarily by increasing the number of functional glucose transport systems. The specific objective for the current year has been to examine, using this assay, various subcellular membrane fractions of the adipose cell as potential sources of those transport systems appearing in the plasma membranes in response to insulin.

Methods: In all experiments, isolated adipose cells were prepared by incubating the whole epididymal fat pads of male Sprague Dawley rats weighing 150-200 µg with collagenase, separating the liberated adipose cells from the stromal-vascular elements by thorough washing, and resuspending the washed cells as a stock cell suspension. Samples of adipose cells from the stock cell suspension were then incubated for varying times at 37°C in the absence or presence of 0.7 or 7.0 nM (100 or 1000 µU/ml) insulin, washed in a tris, EDTA, sucrose buffer, and the homogenized. Various membrane fractions were collected using well differential ultracentrifugation and sucrose gradient techniques, washed, and resuspended in homogenization buffer.

Equilibrium cytochalasin B binding was measured at 4°C by incubating membranes in the presence of increasing concentrations of ^{3}H-cytochalasin B up to 2000 nM and unlabeled cytochalasin B at 0 or 20 µM. The membranes were separated from the incubation medium by centrifugation, and the membrane pellet was determined using U-^{14}C-sucrose.

Glucose transport activity in additional samples of intact adipose cells from the same stock cell suspension was occasionally determined by a ^{14}C-methylglucose uptake technique. Cells were preincubated at 37°C under the

experimental conditions described above, then pulsed for varying times with 0.1 mM ^{14}C-3-O-methylglucose, a nonmetabolizable glucose analogue transported exclusively by the glucose transport system.

Results and Discussion: Initially, isolated rat adipose cells were incubated for 30 min at 37°C in the absence or presence of 7.0 nM insulin. Three subcellular membrane fractions were then obtained: a heavy, particulate fraction containing broken cells, nuclei, mitochondria, and probably lysosomes; a plasma membrane fraction purified on a sucrose step gradient; and a crude microsomal membrane fraction containing all those membranes which do not sediment at 16,0000 x g in 15 min, but do at 160,000 x g in 60 min. While the heavy, particulate fractions from basal and insulin-stimulated cells bind cytochalasin B, the numbers of binding sites/mg protein specifically inhibitable by D-glucose are low and not determinable with any precision. The number of such sites in the plasma membrane fraction, however, is 3-10 pmol/mg membrane protein when prepared from basal cells and, as previously demonstrated, increases 4-5 fold when prepared from cells preincubatedd in the presence of insulin. In contrast, the number of D-glucose-inhibitable cytochalasin B binding sites in the crude microsomal fraction, containing rough and smooth surfaced endoplasmic reticulum and membranes of the Golgi apparatus, is 35-50 pmol/mg protein when prepared from basal cells, and decreases by 40-60% when prepared from insulin-stimulated cells. Differences in the dissociation constants (K_d) of these D-glucose-inhibitable sites for cytochalasin B binding are not observed either among the various membrane fraction or between the basal and insulin-stimulated states.

When marker enzyme activities (5'-nucleotidase and NADH-cytochrome c reductase) are measured in each membrane fraction and the original homogenates and used to correct for cross contamination and the yields of each membrane species, then the respective and reciprocal effects of insulin on the numbers of cytochalasin B binding sites in the plasma and microsomal membrane fractions are stoichiometric. These results suggest that isolated rat adipose cells in the basal state contain an as yet undefined, but large, intracellular pool of glucose transport systems and that insulin triggers some mechanism which translocates transport systems from this pool to the plasma membranes. Since this process occurs as fast as it can be measured, within one min, in the presence of 7.0 nM insulin, this may very well represent the primary mechanism through which insulin stimulates glucose transport in this cell type.

Additional studies are now in progress to further characterize this apparent translocation of glucose transport systems from an intracellular pool to the plasma membrane in response to insulin. First, two subfractions of the microsomal membranes have been prepared which are characterized by an enrichment of marker enzyme activities for membranes of the endoplasmic reticulum and Golgi apparatus, respectively. When these two subfractions

are prepared from basal cells, then the numbers of D-glucose-inhibitable cytochalasin B binding sites/mg protein in the endoplasmic reticulum and Golgi fractions are decreased and increased, respectively, relative to that observed in the crude microsomal membrane fraction previously prepared. Further, insulin pretreatment of the intact cell does not consistently influence of the number of cytochalasin B binding sites in the endoplasmic reticulum fraction, but markedly decreases that in the Golgi fraction. Thus, the insulin-responsive intracellular pool of glucose transporters can be further enriched by partially separating the Golgi membranes from the endoplasmic reticulum. However, the identity of this pool remains to be established.

Second, detailed time course, reversibility, and insulin concentration studies have been undertaken in order to confirm the operation of a translocation process. In the presence of 0.7 nM (100 μU/ml) insulin, the transport response to this hormone in the intact cell occurs with a half-time of approximately 3 min. A similar time course is observed for both the increase in the number of cytochalasin B binding sites in the plasma membrane fraction and the decrease in the number of sites in the Golgi fraction. Further, when insulin-stimulated cells are exposed to excess insulin antibody, the transport response to insulin in the intact cell is rapidly and completely reversed with a half-time of approximately 10 min. The effects of insulin to increase the number of cytochalasin B sites in the plasma membrane fraction and to decrease the number of these sites in the Golgi fraction are similarly reversed. Finally, the progressive stimulatory effect of insulin on transport in the intact cell with increasing hormone concentration is paralleled by progressive effects of insulin on the numbers of cytochalasin B binding sites in the plasma membrane and Golgi fractions. In each case, a one to one correlation can be demonstrated between changes in the numbers of cytochalasin B binding sites in the plasma membranes and in the intracellular pool. The specific steps in this process are currently under investigation.

In collaboration with Lawrence J. Wardzala, an attempt is also presently in progress to examine the glucose transport response to insulin in the rat diaphragm. Preliminary results suggest that a membrane fraction can be prepared from rat diaphragm which is enriched in plasma membranes, that this fraction contains specific D-glucose-inhibitable cytochalasin B binding sites with characteristics similar to those observed in rat adipose cell plasma membranes, and that insulin pretreatment of the intact muscle increases the number of these sites as it increases transport activity in the muscle prior to fractionation. Such studies will establish, at least in part, the generality of the mechanism through which insulin stimulates glucose transport in a variety of insulin-responsive tissues. In addition, similar studies are being extended to isolated adipose cells prepared from human tissue.

Publication:

Cushman, S.W., and L.J. Wardzala. Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell: apparent translocation of intracellular transport systems to the plasma membrane. J. Biol. Chem. 255:4758-4762, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM70503-02 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Alterations in Adipose Cell Function in the Aging Rat Model of Obesity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul J. Hissin	Staff Fellow	CMOS, NIAMDD
OTHER	Ian A. Simpson	Visiting Associate	CMOS, NIAMDD
	Samuel W. Cushman	Associate Chief	CMOS, NIAMDD
	Eddy Karnieli	Visiting Fellow	CMOS, NIAMDD
	Lester B. Salans	Chief	CMOS, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Cellular Metabolism and Obesity

INSTITUTE AND LOCATION
NIAMDD, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.50	1.40	1.10

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Increasing adipose cell size in the aging rat model of obesity is accompanied by an increasing basal glucose transport activity and a decreasing ability of insulin to stimulate glucose transport activity in the intact isolated adipose cell. The ability of insulin to increase the number of functional glucose transport systems in this cell's plasma membranes, as assayed by D-glucose-inhibitable ^{3}H-cytochalasin B binding, is also decreased. Since insulin appears to enhance glucose transport by triggering the translocation of transport systems to the plasma membrane from an intracellular pool which is associated with a fraction enriched in membranes of the Golgi apparatus, the effects of cell size on the number of transport systems in this pool and the total number of transport systems per cell have been examined. The markedly enlarged cells prepared from 800 g rats contain, at most, 50% more transport systems per cell than small cells from 180 g rats. Furthermore, even in the basal state, the number of transport systems in the enlarged cell's plasma membranes is already increased and this cell's intracellular pool is already depleted. Insulin has little further effect on the distribution of transport

Objectives: Increasing adipose cell size in the aging male Sprague Dawley rat model of obesity is accompanied by alterations in adipose cellular metabolic function in vitro which parallel those observed in human adipose cells in vitro in both spontaneous and experimental obesity, as well as the alterations in systemic glucose and lipid metabolism accompanying obesity in man and the aging rat. The overall objective of the present investigation is th describe these alterations in rat adipose cell function in detail and to elucidate the mechanisms responsible for these alterations. Since previous investigations have demonstrated that insulin appears to stimulate glucose transport activity in the rat adipose cell by triggering a rapid and reversible translocation of glucose transport systems from an intracellular pool to the plasma membrane, the specific objective for the current year has been to examine the subcellular distribution of glucose transport systems in the enlarged adipose cells of aged obese rats where basal glucose transport activity is already increased and the transport response to insulin is markedly diminished.

Methods: Small and large isolated adipose cells were prepared by incubating the whole epididymal fat pads of young, lean and old, obese male Sprague Dawley rats with collagenase, separating the liberated adipose cells from the stromal-vascular elements by thorough washing, and resuspending the washed cells as a stock cell suspension. Adipose cell size and concentration in the stock cell suspension were determined using an osmic acid fixation, Coulter Electronic Counter method.

The number of fundamental glucose transport systems was then determined by measuring D-glucose inhibitable equilibrium ^{3}H-cytochalasin B binding to the membranes of the plasma membrane, endoplasmic reticulum, and Golgi fractions prepared from samples of the isolated adipose cells which were first incubated for 30 minutes in the absence or presence of 7 nM (1000 U/ml) insulin. Scatchard analysis of 6 point cytochalasin B binding isotherms in the absence or presence of 500 mM D-glucose permits quantitation of the number of specific cytochalasin B binding sites in the class of sites which has tentatively been identified as the functional glucose transport system. Differences in the number of these sites between the small and large cells were also compared to differences in glucose transport activity in the same preparations of cells by estimating the latter with a ^{14}C-3-O-methyl glucose uptake technique in additional samples of cells from the same stock cell suspensions.

Results and Discussion: Adipose cellular enlargement is accompanied by an increased basal glucose transport activity/cell, but little, if any, change in glucose transport activity/cell in the presence of a maximally stimulating concentration of insulin. The fold response to insulin relative to basal activity is, therefore, decreased. However, when glucose transport activity is expressed per unit cellular surface area, then that in the presence of insulin decreases in parallel with the decreasing fold response.

When plasma membrane, endoplasmic reticulum, and Golgi fractions are prepared from small and large adipose cells following incubation in the absence or presence of insulin, yields of membrane protein are obtained in rough proportion to the surface area of the original intact cells, regardless of hormone exposure. Furthermore, while the activities of enzyme markers such as 5'-nucleotidase, NADH-cytochrome c reductase, and galactosyl transferase do vary with cell size, their distribution among these three fractions does not. However, the number of functional glucose transport systems/mg membrane protein in the basal state is unchanged in the plasma membrane and endoplasmic reticulum fractions, but is markedly decreased in the Golgi fraction from enlarged, relative to small, cells. In addition, while insulin pretreatment of intact small cells increases 4-6-fold the number of glucose transport systems/mg membrane protein in the plasma membrane fraction and decreases by 50-60% the number of systems/mg membrane protein in the Golgi fraction, insulin's corresponding effects in the enlarged cell are markedly decreased.

Measurements of enzyme marker activities in each fraction and in the original homogenates permits a calculation of the cross contamination of membrane species among these three subcellular fractions and the yield of each membrane species during preparation of these fractions. An estimate can thus be made of the number of glucose transport systems in the plasma membranes and intracellular pool of the basal and insulin-stimulated intact cells. In the basal state, the number of transport systems/cell associated with the plasma membranes is increased in the enlarged, relative to the small, cell in rough proportion to this cell's increased surface area, and corresponds to the increased basal rate of glucose transport activity/cell. However, the intracellular pool of transport systems in the enlarged cells is already markedly depleted even in the basal state. Thus the total number of glucose transport systems/ cell appears to remain relatively constant with cellular enlargement while the distribution of transport systems in the enlarged cell in the basal state resembles that observed in the small cell only following insulin stimulation. In addition, the markedly reduced stimulatory effect of insulin on glucose transport activity itself in the intact enlarged cell is closely paralleled by the lack of all but a minimal effect of insulin on the distribution of glucose transport systems between this cell's plasma membrane and its intracellular pool.

A redistribution of glucose transport systems from the intracellular pool to the plasma membrane with cellular enlargement appears to account for the observed increase in basal glucose transport activity/cell. The unchanged density of glucose transport systems/mg plasma membrane protein in the enlarged cell can, in turn, account for the unchanged basal glucose transport activity/unit surface area. Since the total number of glucose transport systems/cell does not appear to increase with cellular enlargement, the intracellular pool becomes depleted and the transport response to insulin is markedly diminished.

Publication:

Foley, J.E., S.W. Cushman, and L.B. Salans. Intracellular glucose concentration in small and large rat adipose cells. Am. J. Physiol. 238: E180-E185, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM70504-02 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Alterations in Adipose cell Function in the Genetically Obese Zucker Fatty Rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Samuel W. Cushman	Associate Chief	CMOS, NIAMDD
	Lester B. Salans	Chief	CMOS, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Cellular Metabolism and Obesity

INSTITUTE AND LOCATION
NIAMDD, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.00	0.00	0.00

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Temporarily inactive.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM70505-02 EFS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Role of Dietary Carbohydrate and Fat in Regulating Rat Adipose Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lester B. Salans	Chief	CMOS, NIAMDD
	Samuel W. Cushman	Associate Chief	CMOS, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Epidemiology and Field Studies Branch

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SUMMARY OF WORK (200 words or less - underline keywords)

Temporarily inactive.

ANNUAL REPORT

OFFICE OF SCIENTIFIC AND TECHNICAL REPORTS

The Office of Scientific and Technical Reports prepares, produces and disseminates information about the varied research and program activities of the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD). This information takes many forms: brochures, pamphlets, fact sheets, background papers, announcements, news releases, reports to Congress, responses to public and Congressional inquiries, exhibits at scientific meetings, and, importantly, contact with print and broadcast media representatives.

NIAMDD extramural programs are organized into four disease areas: Arthritis, Musculoskeletal and Skin Diseases Program; Diabetes, Endocrine and Metabolic Diseases Program; Digestive Diseases and Nutrition Program; and Kidney, Urologic and Blood Diseases Program. Information responsibilities are divided along these lines, with an information specialist designated to cover the activities of each cluster. Intramural research, with the exception of the diabetes branch, is assigned to a writer-editor, who is also the correspondent for the NIH Record.

PUBLICATIONS PROGRAM

During fiscal year 1980, OSTR initiated a "series" approach to the publications program. A unified graphic design was developed for each of four series of publications: brochures, pamphlets, fact sheets and reports. The new series was inaugurated with the publication of the pamphlet Human Growth Hormone, aimed at a lay audience, and a companion booklet for the scientist, entitled Research Availability of Anterior Pituitary Hormones. Both were distributed at the October 1979 meeting of the American Society of Clinical Pathologists.

A new pamphlet addressing the important subject of insulin-dependent diabetes, formerly referred to as juvenile diabetes, was produced and distributed at the June 1980 meetings of the Juvenile Diabetes Foundation and the American Diabetes Association.

A special report, Diabetes Mellitus: Trans-NIH Research 1980, was published to highlight research advances over the past year. A Report of NIH Diabetes Mellitus Coordinating Committee was produced by NIAMDD to highlight programmatic initiatives in diabetes.

Fact sheets were prepared on Foot Care for the Diabetic and Diabetes Overview, each for distribution to the lay public. All publications in the diabetes area were also distributed through the National Diabetes Information Clearinghouse of the Institute.

New publications on cystic fibrosis included NIAMDD 1980: Cystic Fibrosis, a report on research advances in the field, and a three-volume series, Cystic Fibrosis: Model Systems, aimed at a scientific audience.

In the digestive diseases area, fact sheets on ulcerative colitis and primary biliary cirrhosis were produced. A report on research advances, NIAMDD 1980: Digestive Diseases, was published for distribution at scientific meetings.

A fact sheet on intramural research on lymphapheresis as a possible treatment for rheumatoid arthritis was published following widespread media coverage of the highly experimental procedure.

Reports on arthritis included the Third Annual Report of the Director on the Arthritis Program and NIAMDD 1980: Arthritis, and Related Rheumatic Diseases. Arthritis publications, including approximately 6,000 copies of How To Cope With Arthritis, were distributed through NIAMDD's Arthritis Information Clearinghouse.

Research results were published in the report NIAMDD 1980: Kidney and Urinary Tract Diseases and Treatment. This report and other scientific publications were distributed at major medical and scientific meetings.

Particularly worthwhile publications of nongovernmental organizations are purchased by OSTR to supplement existing educational materials. Reprints of Consumer Reports' two-part series on arthritis were obtained for distribution by OSTR.

Two brochures -- Human Growth Hormone and Peptic Ulcer -- won Blue Pencil Publication Awards from the National Association of Government Communicators.

Approximately 68,000 publications were distributed to the public during the period July 1979 to May 1980.

CONGRESSIONAL INQUIRIES

The Office of Scientific and Technical Reports handles constituent correspondence referred from the Congress and the White House. Because of active public interest in a wide-ranging number of subject areas within its research purview, NIAMDD receives a large volume of these requests.

During the period July 1979 through May 1980, OSTR prepared 156 written responses to inquiries from Senators, Congressmen, the Secretary, DHHS, and the White House. The OSTR also received 283 telephone inquiries from Congressional staff members.

Most Congressional inquiries concern the funding of individual grants and specific research programs; lobbying efforts of numerous voluntary health agencies; proposed "cures" for various ailments; the Federal funding status of a particular research area; requests from constituents for financial assistance; and requests for information from ill constituents, their families and friends.

Overall, written responses to Congress and the White House during 1979-80 increased by 14 over the previous year.

OSTR staff members prepared comprehensive special reports on five separate research areas in preparation for Congressional appropriation hearings. The reports covered research advances in arthritis, diabetes, cystic fibrosis, digestive diseases, and kidney and urinary tract diseases. As indicated previously, these reports were later published as a series and distributed to relevant publics.

In addition, OSTR prepared the budget narrative and the director's opening statement to Congress, highlighting selected research findings of Institute scientists and grantees.

PUBLIC INQUIRIES

A significant portion of the U.S. public is directly concerned with the disease areas under investigation by NIAMDD. An estimated 60 million people in the U.S. are victims of arthritis, diabetes and digestive diseases alone. During the period July 1979 through May 1980, OSTR received approximately 21,206 telephone inquiries from patients, their relatives and friends, students, allied health professionals, physicians and scientists. In addition, 240 letters were written in response to public inquiries, exclusive of requests for publications or Congressional inquiries.

Requests for information dealt with questions about controversial forms of therapy, how to become a patient in an NIAMDD research study, financial aid, physician referral, accepted diagnosis and treatment procedures, proposed "cures" for certain diseases, and NIAMDD research grant and contract programs.

PRESS ACTIVITIES

Contact with representatives of print media is an integral part of OSTR's public information program. In support of the established policy to publicize NIAMDD's programs and activities, OSTR reaches a variety of media outlets through personal contact and distribution of news releases and announcements.

Arthritis research supported by NIAMDD was featured in two major magazine articles in October 1979: "Arthritis: New Treatment, New Drugs, New Hope," Family Circle, and "Arthritis: An encouraging progress report," Better Homes and Gardens. A bylined article on rheumatoid arthritis by Dr. G. Donald Whedon appeared in the nationally-circulated Pharmacy Times in September 1979.

Lymphapherisis, the experimental technique for treatment of severe rheumatoid arthritis, was the subject of a Time magazine article, July 9, 1979. The subject was also covered by Medical World News in September 1979.

New findings on Lyme arthritis, now termed Lyme disease, were covered in the May 1980 issue of the award-winning Research Resources Reporter, published by the Division of Research Resources.

Newsweek magazine (February 4, 1980) covered the research of NIAMDD grantee Dr. Andrew Bassett into the electrical stimulation of

bone growth. Major articles on the bone diseases scoliosis and Paget's disease appeared in the mass circulation Sunday supplement, Parade.

A major article on the skin disease vitiligo, and NIAMDD grantee Dr. John A. Kenney, Jr., appeared in the Washington Post on October 10, 1979. Vogue magazine quoted NIAMDD dermatology program director Dr. Laurence Miller in its May 1980 health section article on the use of cortisone in treating skin diseases.

NIAMDD's field study of obesity and diabetes in the Pima Indians was the subject of a feature article in the New York Times on February 5, 1980. The project was also the subject of a cover story in Medical World News on March 3, 1980.

Diabetes research at NIH was the subject of a feature article in the New York Times on August 5, 1979, and also the cover story of Medical World News' October 29, 1979, issue.

A bylined feature article by Dr. G. Donald Whedon appeared in the nationally-circulated Pharmacy Times. Staff member Patricia Sheridan coauthored the article, entitled "What You Should Know About Diabetes Research."

The Chicago Tribune ran a six-part series on digestive diseases with frequent reference to research supported by NIAMDD. An article on ulcers and related research, referencing NIAMDD, appeared in the June 1980 issue of Better Homes and Gardens. Staff member Irving Shapiro and Dr. Harold Roth also wrote an article on peptic ulcer, which appeared in the June issue of Occupational Health & Safety Magazine.

A feature article on liver disease, referencing NIAMDD and grantee research, appeared in the November 12, 1979, issue of Newsday.

In November, OSTR coordinated the announcement of the presentation of the Lita Annenberg-Hazen Award to Dr. Jesse Roth, chief of the intramural diabetes branch, with the Public Affairs Office of Mt. Sinai Hospital in New York. Correspondents from the New York Times, New York Daily News, Medical World News, NBC and ABC covered the event.

OSTR prepared a number of news releases during the past year, including announcements on the appointment of a new information officer at NIAMDD, results of a 5-year study on the treatment of Crohn's disease, appointment of four members to the NIH Advisory Arthritis, Metabolism, and Digestive Diseases Council, progress in diabetes research, and presentation of the Lita Annenberg-Hazen Award to Dr. Jesse Roth.

Articles on NIAMDD research appeared in the prestigious "From the NIH" column in the Journal of the American Medical Association. Topics included psoriasis, diabetes classification, and an overview of diabetes research.

Various information materials for the research community and the news media were prepared for the Chronic Renal Disease Conference in January

1980 and for a consensus development conference on the use of endoscopy in upper gastrointestinal bleeding scheduled for August 1980.

RADIO AND TELEVISION

A number of NIAMDD scientists were featured this year on radio and television broadcasts, both locally and nationally. Dr. Denis McCarthy discussed ulcers on the local Channel 9 Panorama program in February 1980. Dr. Laurence Miller also appeared on the program to speak about sunburn and related skin disorders in June 1980.

Dr. Nancy Cummings, director of the Kidney, Urologic and Blood Diseases Program, appeared in January on the Doctor's House Call segment of the Channel 7 program AM Washington. She was also interviewed on the Fred Fiske Show, WAMU-FM, and was one of several NIH scientists interviewed by the Canadian Broadcasting Company for a program on NIH research.

Dr. Lester Salans, associate director for diabetes, endocrine and metabolic diseases, was a featured guest in February on the nightly syndicated radio talk show hosted by Dr. Gabe Mirkin. Dr. Salans also appeared on a WGAY radio talk show.

Dr. Harold Roth, associate director of the Digestive Diseases and Nutrition Program, appeared on radio and television programs to discuss ulcers and other digestive problems, as well as the activities of the National Commission on Digestive Diseases.

Taped interviews, which were aired locally and distributed to radio stations throughout the country, featured Dr. John Decker, Dr. Harold Roth, Dr. Lester Salans, and Dr. Denis McCarthy.

NIAMDD also supported the production of a half-hour educational film on cystic fibrosis. The film, which includes an interview with an NIAMDD grantee, will be distributed nationally by the Cystic Fibrosis Foundation.

EXHIBITS

OSTR has responsibility for coordinating the exhibit program for NIAMDD, designed primarily for a scientific audience. Eight major exhibits were viewed at various medical and scientific meetings throughout the U.S. by approximately 75,000 physicians, scientists and allied health professionals.

Institute exhibits on artificial kidney research, human growth hormone, cystic fibrosis, digestive diseases, diabetes, and general research were displayed at meetings such as those of the National Kidney Foundation, American Society for Artificial Internal Organs, American Society of Nephrology, Federation of American Societies for Experimental Biology, American Gastroenterology Association, American Diabetes Association, Juvenile Diabetes Foundation, American Thoracic Society, Endocrine Society, New York State Medical Society, and College

of American Pathologists. Appropriate publications were distributed by OSTR staff members who accompanied exhibits.

OSTR staff supported the EEO office by producing an exhibit and attending the Eighth Annual Minority Biomedical Research Symposium, April 7-10, in Atlanta, Ga.

During the past year, the use of "crated" exhibits has been supplemented by the addition of portable displays. Individual panels that are easily updated have been designed for the cystic fibrosis program, the endocrinology program, the digestive diseases program, the diabetes program, and the overall NIAMDD extramural program. The exhibits program continues to be a valuable method of reaching a primary audience of NIAMDD, the scientific and medical community. Thousands of extramural publications, program announcements, and other Institute materials are distributed directly to relevant publics at meetings each year.

NIH RECORD

A primary method of communication is through the NIH Record, which is distributed to every NIH employee and to wire services, local news media, science writers and editors, and scientists in the U.S. and abroad. Because of the importance of this publication in reaching NIAMDD's relevant publics, a writer-editor was assigned as NIH Record correspondent in anticipation of providing greater visibility for NIAMDD's programs, particularly intramural activities. As a result of this emphasis, the number of articles on NIAMDD activities increased from 16 in 1978-79 to 40 in 1979-80.

NIAMDD news releases, described earlier, are published by the NIH Record. In addition, during the past year articles were written for the Record on the following subjects: the 75th anniversary of the Laboratory of Chemistry; the first anniversary of the cytopheresis laboratory of the Arthritis and Rheumatism Branch; Dr. Edwin Becker, chief of the Laboratory of Chemical Physics, named acting director of the Fogarty International Center; Ms. Betsy Singer named public information officer; Mr. J. Harrison Ager, EEO coordinator, reappointed to the Maryland State Board of Higher Education; and Dr. Edwin Becker, chief of the Laboratory of Chemical Physics, named NIH Associate Director for Research Services.

NIAMDD personnel received awards that were subjects of the following Record articles: Drs. Jesse Roth and Helena Wachslicht-Rodbard presented with the third annual Berson-Yalow Award; Mrs. Lillian Perry presented with the HEW Merit Award; Dr. Walter Gilbert, NIH lecturer, presented with Albert Lasker medical research prize and the Gairdner Foundation Award; Dr. Donald M. Jerina presented with the 1980 Hillebrand Award by the Chemical Society of Washington; Dr. Bruce D. Weintraub presented with the 1979 Van Meter-Armour Prize by the American Thyroid Association; Dr. Sanford M. Rosenthal, scientist emeritus, presented with the 1980 ASPET Award for experimental therapeutics by the American Society for Pharmacology and Experimental Therapeutics; Dr. Paul di Sant'Agnese presented with the first Medical Award of Merit by the Cystic Fibrosis Foundation; Dr. Jesse Roth awarded the PHS Distinguished Service Medal; and Mr. Walter Stewart presented with an

award by the Secretary of Commerce for work culminating in the synthesis of certain yellow dyes.

During the past year, articles also covered elections to scientific organizations such as the following: Dr. Gerald F. Combs, nutrition program director, named president of the Federation of American Societies for Experimental Biology; Dr. Sanford M. Rosenthal elected to the American Academy of Arts and Sciences; and Dr. Joseph E. Rall, intramural program director, elected to the National Academy of Sciences.

Conferences and seminars covered in the NIH Record included: Science Writer's Seminar on nutrition research featuring lecture by Dr. Herbert G. Windmueller; the 3-day Chronic Renal Disease Conference; NIAMDD Secretaries' Week Seminars; and Combined Clinical Staff Conference on Inappropriate Secretion of Thyrotropin.

NIAMDD participation in the prestigious NIH lecture series included Dr. Walter Gilbert's lecture on November 28, 1979, and that of Dr. Baruch Blumberg, Nobel laureate, on May 14, 1980.

NIAMDD research results received coverage in the following articles: immune Interferon found in the blood of patients with autoimmune disease; hGH synthesized using genetic engineering techniques; students study diabetes mellitus in the laboratory of Dr. Jesse Roth's diabetes branch; laboratories at NIH perform valuable research; acid proteases studied using crystallographic techniques; and zinc-fortified foods prevent nutritional defects.

Several NIAMDD-sponsored publications received notice in the NIH Record, including the 2nd Annual Report of the Director NIAMDD on the Arthritis Program; the major report entitled Analysis of Research Needs and Priorities in Dermatology; Volume 3 of the series Cystic Fibrosis: A Disease in Search of Ideas; and the "Proceedings of the Consensus Conference on Morbid Obesity," published in The American Journal of Clinical Nutrition. Articles marked the retirements of the following individuals: Mr. Dave Rogerson, head of the pilot plant of the Laboratory of Nutrition and Endocrinology; Mr. Linden Neff, grants management officer; and Mr. Milton Parker, biologist.

The deaths of two former NIAMDD employees received coverage in the Record. They were Dr. Ralph D. Lillie, retired chief of the Laboratory of Pathology and Biochemistry, and Mrs. Florence J. Hooker, retired intramural senior secretary.

RESEARCH REPORTS

Research reports are brief summaries about significant findings reported in the literature by NIAMDD scientists and grantees. Prepared by OSTR, these reports reflect the scope of the Institute's research programs, and are used primarily as a basis for special reports to Congress and related reports to DHHS and NIH.

The following examples are representatives of the 33 research reports prepared during the past year: 1) insulin resistance of obese subjects is overcome when maximal plasma insulin levels normalize blood sugar; 2) elevated serum pepsinogen I may be a genetic marker for susceptibility to duodenal ulcer disease; 3) gold compounds alter the structure and functions of cultured human monocytes; 4) high carbohydrate, high plant fiber diets may reduce insulin requirements of maturity-onset diabetics; 5) combinations of the cigarette smoke components nicotine and carbon monoxide induce profound tissue and systemic changes in rats; 6) plasmapheresis and immunosuppressive drugs reverse kidney lesion in Goodpasture's syndrome; 7) two influenza vaccines proved to be safe and effective in rheumatic disease patients; 8) a direct link was found between the degree of blood sugar elevation and impairment of peripheral nerve function in advanced diabetes in man; 9) physical training increases tissue sensitivity to insulin and insulin-binding to body cells; 10) the antiulcer drug cimetidine exerts an antifertility side effect in men via a hypothalamo-pituitary-testicular mechanism.

SPECIAL PROJECTS

OSTR is leading activities surrounding the celebration of NIAMDD's 30th anniversary on October 15, 1980. A full-day symposium, entitled "DNA, the Cell Nucleus and Genetic Disease," is being sponsored by NIAMDD's intramural research program at Masur Auditorium, followed by a dinner at the Naval Medical Center, Bethesda, Maryland.

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